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14. ABSTRACT

The majority of breast tumors express the estrogen receptor α (ERα), which plays important roles in breast cancer pathogenesis and progression, and hormonal therapies, such as tamoxifen, are the first line of adjuvant therapy (1, 2). Unfortunately, half of these patients will ultimately fail therapy due to de novo or acquired resistance. Moreover, patients with ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2) triple negative breast cancer (TNBC), which is aggressive with high recurrence, metastatic, and mortality rates (3), do not respond to hormonal therapies and have limited treatment options. Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis (4), which may be due in part to the high frequency of TNBC and ineffectual hormonal therapy (5). However, the links between obesity and breast cancer are not understood and is the focuse of our study. As hormonal therapy is so effective with relatively few side effects, the possibility of reversing hormonal unresponsiveness is an appealing treatment approach. Our study will lead to novel therapies that will overcome the overarching challenges of developing safe and effective drugs for treating obesity-promoted cancers and TNBC and will identify the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), produced by sphingosine kinases (SphK1 and SphK2), as a critical factor that links obesity and chronic inflammation to drive breast cancer growth and metastasis.

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1. INTRODUCTION

The majority of breast tumors express the estrogen receptor α (ERα), which plays important roles in breast cancer pathogenesis and progression, and hormonal therapies, such as tamoxifen, are the first line of adjuvant therapy (1, 2). Unfortunately, half of these patients will ultimately fail therapy due to *de novo* or acquired resistance. Moreover, patients with ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2) triple negative breast cancer (TNBC), which is aggressive with high recurrence, metastatic, and mortality rates (3), do not respond to hormonal therapies and have limited treatment options. Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis (4), which may be due in part to the high frequency of TNBC and ineffectual hormonal therapy (5). However, the links between obesity and breast cancer are not understood and is the focuse of our study. As hormonal therapy is so effective with relatively few side effects, the possibility of reversing hormonal unresponsiveness is an appealing treatment approach. Our study will lead to novel therapies that will overcome the overarching challenges of developing safe and effective drugs for treating obesity-promoted cancers and TNBC and will identify the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), produced by sphingosine kinases (SphK1 and SphK2), as a critical factor that links obesity and chronic inflammation to drive breast cancer growth and metastasis.

2. KEY WORDS

sphingosine-1-phosphate, sphingosine kinase, FTY720 (fingolimod, Gilenya), triple negative breast cancer, ERα, obesity, histone deacetylase, inflammation

3. ACCOMPLISHMENTS

3.1. Major Goals of the Project

Our project has three major aims.

Aim 1. Determine the role of SphK1 and S1P in obesity promoted chronic inflammation and tumor progression and decipher the molecular links between the SphK1-S1P-S1PR1 axis and persistent NF-κB and STAT3 activation.

Aim 2. Target the SphKs/S1P/S1PR1 axis with fingolimod/FTY720 for treatment of obesity associated breast cancer to suppress the malicious amplification cascade, and reactivate ER expression in ER-negative breast cancer.

Aim 3. Examine the association of the SphKs/S1P/S1PR1 axis in human breast cancer and prognosis.

The inability to effectively predict, prevent, and treat metastatic breast cancer is a major problem in breast cancer care. This proposal will provide evidence that the SphKs-S1P-S1PR1 axis is one of the critical factors that bridges obesity, chronic inflammation, and breast cancer and will pave the way for development of new adjuvant therapies targeting this axis as a promising strategy for effective treatment of advanced and refractory breast cancer.

3.2. Accomplishments Under These Goals and Significant Result

<u>Aim 1.</u> Role of SphK1 And S1P In Obesity Promoted Breast Cancer Progression

HFD-induced obesity increases breast cancer growth, S1P, tumor associated macrophages, and cytokines in immunocompetent mice

To investigate the mechanisms underlying the promotion of breast cancer progression by obesity, E0771 mouse breast cancer cells were implanted into mammary fat pad of syngeneic C57Bl/6 mice, which were fed with HFD or chow diet for 12 weeks prior to the implantation. The mice fed with HFD developed significantly larger tumors within 30 days than those on chow diet. Moreover, HFD also increased tumor associated macrophages, which are known to have important roles in obesity-related cancer progression and the expression of the pro-inflammatory cytokines, IL-6 and TNF-α, produced by them. Since we previously showed that the bioactive sphingolipid metabolite S1P links inflammation and cancer progression, and stimulates tumor-associated inflammation and increases cytokines such as IL-6 and TNF-α (6), we investigated the role of S1P in obesity-related inflammation and cancer progression. We found that expression of SphK1 and S1PR1 was increased in the tumors from mice fed HFD. Importantly, HFD also increased S1P in mammary fat pads and in breast tumors. There was also a greater increase of S1P in the circulation and in the lungs of tumor bearing mice fed with HFD than those fed with chow diet.

FTY720 attenuates obesity-induced tumor progression, S1P, and inflammation

FTY720 (fingolimod, Gilenya), an FDA approved pro-drug for the treatment of multiple sclerosis, is phosphorylated *in vivo* by sphingosine kinase 2 (SphK2) to its active form FTY720-phosphate (FTY720-P), a mimetic of sphingosine-1-phosphate (S1P) and an agonist of four S1P receptors (S1PRs) that interferes with immune cell trafficking by inducing internalization and degradation of S1PR1 (7). However, FTY720 has strong anticancer effects *in vitro* and *in vivo* in various types of cancers including breast (8, 9) that are not well understood.

In syngeneic mice implanted with E0771 breast cancer cells, daily oral dosing of FTY720 (1 mg/kg/day) significantly suppressed tumor progression determined by decreases of primary tumor volume and tumor weight in HFD fed obese mice. FTY720 administration however did not significantly affect weight gain of HFD fed mice indicating that FTY720 did not affect diet intake. Moreover, FTY720 treatment also significantly reduced tumor associated macrophages and levels of S1P and the pro-inflammatory cytokines TNF-α and IL-6 in the tumors of HFD-fed mice.

<u>Aim 2.</u> Targeting the SphKs/S1P/S1PR1 axis with fingolimod/FTY720 for treatment of obesity associated breast cancer.

The pro-drug FTY720/fingolimod is phosphorylated by SphK2 in the nucleus of breast cancer cells; the active phosphorylated FTY720 inhibits class I HDACs and enhances histone acetylations and regulates gene expression independently of S1PRs

Because SphK2, the main SphK isoenzyme that phosphorylates FTY720 is predominantly in the nucleus (10), we asked where FTY720 is phosphorylated in breast cancer cells. We found that FTY720 is phosphorylated in human and mouse breast cancer cells by nuclear SphK2 and accumulates there. Moreover, nuclear FTY720-P potently inhibited class I histone deacetylases (HDACs) and enhanced histone acetylations and gene expression in breast cancer cells. To

conclusively demonstrate that the effects on histone acetylation are due to direct intranuclear action of FTY720-P on HDAC activity independently of canonical signaling through S1P receptors (S1PRs), experiments were carried out with purified MCF7 nuclei devoid of S1PRs. FTY720-P, more potently than S1P enhanced histone acetylation in these nuclei. Microarray analysis also indicated that the effects of FTY720 on gene expression were clearly distinguished from S1P receptor occupancy. Unsupervised cluster analysis revealed that there were no major differences in the clustering of the gene expression profiles between vehicle and S1P treated groups, whereas major differences were observed in gene clustering between them and the FTY720 and SAHA treated groups. Although activation of S1PRs did not alter gene expression, FTY720 treatment changed the profiles of 713 genes. In comparison, the pan HDAC inhibitor SAHA altered expression of 3166 genes, of which 276 were common to FTY720. GO analysis revealed that the majority of the commonly affected genes were related to transcription followed by lipid and steroid biosynthesis, transport, metabolic processes, cell growth and angiogenesis genes. These results indicate that breast cancer cells take up FTY720 and that FTY720-P produced in the nucleus by SphK2 inhibits class I HDACs and increases specific histone acetylations and regulates gene expression independently of S1PRs.

FTY720 treatment suppresses development and progression of spontaneous breast tumors in high fat diet fed MMTV-PyMT transgenic mice

Aberrant expression of class I HDACs has been found in breast cancers, and HDAC inhibitors are in clinical trials for breast cancer treatment (11-14). Therefore, we examined whether the HDAC inhibitory activity of FTY720-P could mitigate breast cancer development. We used female MMTV-PyMT transgenic mice, which spontaneously develop breast cancer that closely mimics progression of the human disease (15, 16). These transgenic mice on a normal chow diet spontaneously developed palpable mammary tumors by 7.5 weeks. Feeding a HFD accelerated the onset of tumors that were palpable by 6 weeks and increased tumor multiplicity and size (Fig. 1A). FTY720 administration increased the latency for appearance of palpable tumors and dramatically suppressed tumor development in mice on HFD (Fig. 1A). Consistent with the profound effect on tumor size, FTY720 decreased HFD-induced proliferation determined by Ki67 staining (Fig. 1A insert).

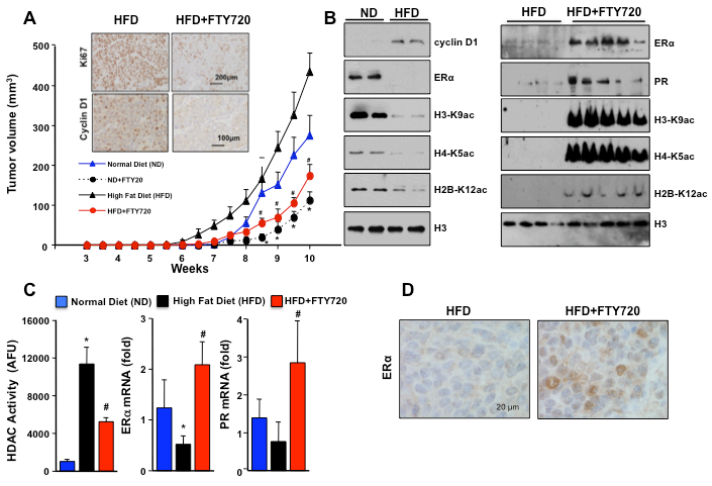

Figure 1. FTY720 suppresses advanced tumorigenic mammary lesions in HFD fed PyMT transgenic mice and reverses HFD-induced loss of estrogen and progesterone receptors. Female PyMT transgenic mice were fed with a normal diet (ND) or a Western high fat diet (HFD), and were treated daily with saline or FTY720 (1 mg/kg) by gavage starting after weaning. (A) Tumor volumes were determined at the indicated times. Insert, Ki67 staining, (B) Nuclear extracts from tumors were analyzed by western blotting with the indicated antibodies. (C) HDAC activity and mRNA levels of ERα and PR in tumors. Data are mean ± SEM. *, $P < 0.05$ compared to ND; #, $P < 0.05$ compared to HFD. (D) Representative images of tumor sections immunostained with anti-ERα.

FTY720 treatment reverses HFD-induced loss of estrogen and progesterone receptors in advanced carcinoma

In agreement with the advanced carcinoma observed in animals fed with HFD, expression of cyclin D1 was elevated (Fig. 1B). In contrast, ERα protein and mRNA as well as PR mRNA were significantly reduced (Fig. 1B,C), which in human breast cancers are associated with poor prognosis (17). Notably, these characteristics of advanced tumorigenic mammary lesions were reversed by FTY720 treatment of HFD fed transgenic mice (Fig. 1B,C). Intriguingly, HFD reduced acetylation of histones in breast tumors that corresponded with increased nuclear HDAC activity (Fig. 1B,C). Conversely, FTY720 increased these specific histone acetylations concomitantly with reduced HDAC activity (Fig. 1C) and importantly, increased expression of ERα and PR (Fig. 1B-D).

FTY720 sensitizes triple negative breast cancer cells to tamoxifen by reactivation of silenced ERα expression

HDACs are negative regulators of the ERα transcriptional complex and HDAC inhibitors have been shown to epigenetically restore ERα expression and reverse tamoxifen (TAM) resistance in hormone resistant breast cancer cells (18-21) and in preclinical animal studies (22, 23). Therefore we asked whether similar to other HDAC inhibitors, FTY720 treatment might also affect ERα re-expression in ERα negative breast cancer. Similar to SAHA, FTY720 also enhanced expression of ERα and PR in ERα negative human MDA-MB-231 and mouse 4T1 breast cancer cells (Fig. 2A). Neither SAHA nor FTY720 had a significant effect on expression of ERβ (Fig. 2B). Moreover, ChIP assays showed that FTY720, even more potently than SAHA, enhanced association of acetylated histone H3 at the ERα promoter (Fig. 2B), indicating that reactivation of ERα expression correlates with ERα promoter hyperacetylation. Therefore, we next examined whether FTY720 could also induce sensitivity to TAM, an ERα antagonist. As expected, TAM alone at concentrations up to 10 μM did not inhibit growth of MDA-MB-231 cells (Fig. 2C). Like other HDAC inhibitors (19-21), FTY720 reduced growth in a concentration-dependent manner and sensitized the cells to TAM. Similarly, in highly metastatic 4T1 murine mammary carcinoma cells, FTY720 also greatly enhanced the growth inhibitory effect of TAM with a Synergistic Index of 0.23.

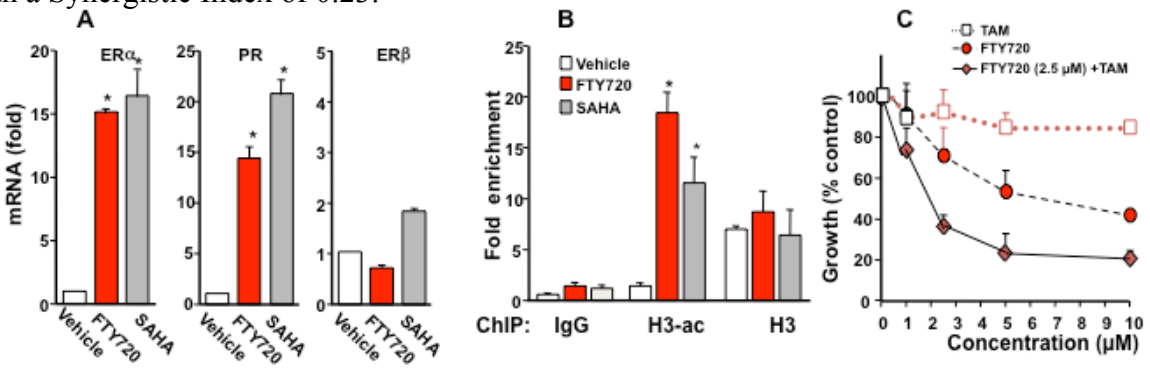

Fig. 2. FTY720 induces ERα expression in ERα-negative human and murine breast cancer cells and sensitizes them to tamoxifen. MDA-MB-231 cells were treated with FTY720 (5 μM) or SAHA (1 μM) for 24 h. ERα, PR, and ERβ mRNA levels were determined by QPCR. (B) Cells were subjected to ChIP analyses with antibodies to H3-ac, H3, or normal rabbit IgG, as indicated. Relative binding to the promoter is expressed as fold enrichment compared to input. Data are mean ± SD. *P < 0.003 compared to vehicle. (C) MDA-MB-231 cells were treated with the indicated concentrations of TAM or FTY720, or with 2.5 μM FTY720 with increasing concentrations of TAM for 48 h and cell proliferation determined. Data are expressed as % of untreated control.

FTY720 increases therapeutic sensitivity of ERα negative syngeneic breast tumors to tamoxifen

As we have found that FTY720 treatment induces functional ERα reactivation and sensitizes ERα negative breast cancer cells to TAM *in vitro*, we next determine whether FTY720 also enhances anti-estrogen therapy *in vivo*. We utilized a syngeneic mouse metastatic breast cancer model, instead of conventional xenografts in immune compromised nude mice, that more accurately mimics human breast cancer (24, 25). ERα-negative 4T1 cells were orthotopically implanted into the second mammary fat pad of immune-competent mice. 4T1 cells produced large primary tumors in the chest mammary fat pad that were not significantly reduced by TAM (Fig. 3A). Orally administered FTY720 reduced tumor growth, an effect that was significantly potentiated by co-administration of TAM (Fig. 3A). Remarkably, FTY720 enhanced the anti-tumor efficacy of TAM more than SAHA. In agreement with the decreased nuclear HDAC activity in tumors from animals treated with FTY720 or SAHA (Fig. 3B), expression of ERα was increased in tumors from FTY720 treated mice that was more prominent when combined with TAM than even in tumors from mice treated with the combination of SAHA and TAM (Fig. 3C). Our data suggest that FTY720 induces epigenetic ERα re-activation *in vivo* to enhance hormonal therapy of ERα negative breast cancer.

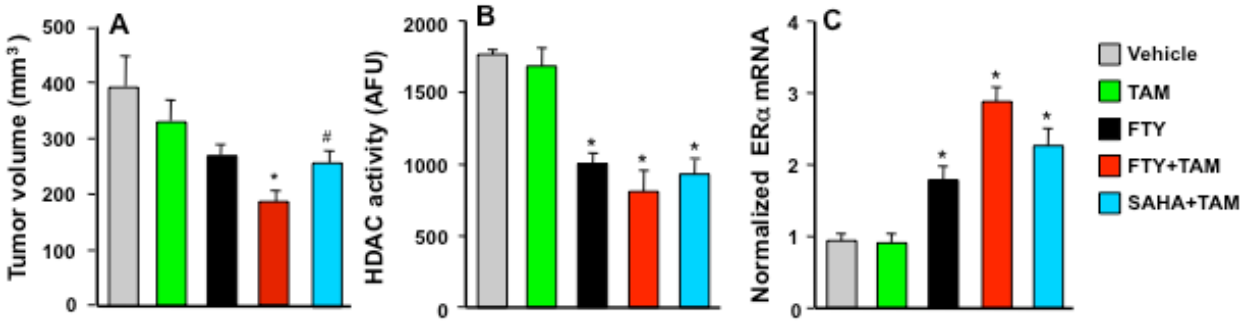

Fig. 3. FTY720 reduces breast tumor growth and enhances anti-cancer effectiveness of TAM in ERα-negative 4T1 syngeneic xenografts. 4T1 cells were surgically implanted into the 2nd mammary fat pads. Tumor-bearing mice were randomized into 5 groups 2 days after implantation and then treated with vehicle, FTY720 (1 mg/kg), TAM (25 mg/kg), FTY720 plus TAM or SAHA (i.p 20 mg/kg) plus TAM by gavage daily till day 15. (A) Tumor volumes on day 15. (B) HDAC activity in nuclear extracts of tumors was determined. (C) Expression of ERα in the tumors was analyzed by QPCR and normalized to Gapdh. Data are mean ± SEM. *, $P < 0.01$, #, $P < 0.05$ compared to Vehicle.

3.3. Opportunities for Training and Professional Development

Although the project was not designed to provide training and professional development opportunities, we should point out that the VCU School of Medicine developed several new programs for enhancing training and professional development of graduate students and postdoctoral fellows in recognition of the important roles they fulfill. The Office of Postdoctoral Services at VCU provides postdoctoral fellows with career and mentoring resources including FASEB Individual Development Plan, Individual Development Plan web-based tool, career Information from the National Postdoc Association, job opportunities in BioCareers, career resources from AAAS, CV/resume writing and samples from UCSF Office of Career and Professional Development, career development websites. For graduate students, these functions reside within the Office of Graduate Education. While no graduate students were included in the

original proposal, Melissa Maczis, who rotated in my lab during her first year as a PhD student, recently decided to join my lab. Although she is not supported by this DoD grant as the VCU School of Medicine is supporting her for an additional year, I have already begun advising her on career development. Using the "my Individual Development" plan website, she created an Individual Development Plan (IDP) she is using to record the immediate and long term objectives of her research and plan of her career path.

3.4. How were the results disseminated to communities of interest

We presented several research lectures on this project to the cancer research community at the Massey Cancer Center in retreat and in the regular meeting of the Massey Cancer Center Cancer Cell Signaling Program. We also presented this work to the international scientific community.

Dr. Hait presented: The Phosphorylated Pro-drug FTY70, a Histone Deacetylase Inhibitor, Reactivates ERα Expression and Enhances Hormonal Therapy of ERα-Negative Breast Cancer. MCC, Richmond, VA. February 20, 2015.

Dr. Spiegel presented: Sphingosine-1-phosphate, from insipid lipid to key regulator of lymphocyte trafficking and the link between inflammation and cancer. Sackler Lectureships, Tel-Aviv University, Tel Aviv, Israel. March 15, 2015

Dr. Spiegel presented: Novel actions of Sphingosine-1-phosphate and the multiple sclerosis pro-drug FTY720/fingolimod: Implications for cancer. Sackler Lectureship, Tel-Aviv University, Israel. March 18, 2015.

Dr. Spiegel presented: Women in Science. Cell Biology of Animal Lectins. The Weizmann Institute of Science, Rehovot, Israel. June 21-25, 2015.

Dr. Spiegel presented: Active Phosphorylated FTY720/Fingolimod is a Potent Inhibitor of Class I Histone Deacetylases that Reactivates Estrogen Receptor Expression and Increases Hormonal Therapeutic Sensitivity of Breast Cancer. International Ceramide Conference Sphingolipid Club Joint Meeting, Cesme, Izmir, Turkey. May 6-10, 2015.

Dr. Spiegel presented a Keynote Address as a JLR Special Lecture: Sphingosine1phosphate from Bench to Clinic: Evolving concepts. FASEB Science Research Conference - Lysophospholipids and related mediators - From bench to clinic. Banff, Alberta, Canada. August 23-28, 2015.

3.5 The Plan for the Next Reporting Period

Continue as was proposed in the original application. As mentioned above, we have already made good progress in Aim 1 and substantial progress in Aim 2. In the next period, we intend to begin Aim 3 and continue to accomplish Aim 1 and Aim 2.

4. IMPACT

4.1. The impact on the development of the principal discipline of the project

Hormonal therapies, including selective estrogen receptor modulators and aromatase inhibitors, are the standards of care for treatment of ER positive breast cancer. However, development of resistance to hormone therapies in advanced breast cancer is a major obstacle. Moreover, treatment of TNBC, which has poor prognosis, remains challenging because the tumors are more aggressive and resistant to hormonal therapy (17). Obesity, which has drastically increased in the last decades, has been associated with increased risk for lymph node metastasis, endocrine therapy resistance, larger tumors, death, and for presenting with TNBC (4,

5, 26-29). Several HDAC inhibitors have been developed that restored the efficacy of hormonal therapy in preclinical models (22, 23, 30) and a few have advanced to clinical trials (31, 32), and to a phase III clinical trial that is currently underway (Clinical-Trials.gov identifier: NCT02115282).

In this study we have shown that the pro-drug FTY720 (fingolimod, Gilenya) approved for human use is phosphorylated to the active form, FTY720-P, a histone deacetylase inhibitor that reactivates ERα expression and enhances hormonal therapy for breast cancer. Moreover, oral administration of clinically relevant doses of FTY720 suppressed development, progression, and aggressiveness of spontaneous breast tumors in transgenic mice on HFD and reversed HFD-induced loss of estrogen and progesterone receptors in advanced carcinoma. Our work suggests that a multi-pronged attack with FTY720 is a novel combination approach for effective treatment of conventional hormonal therapy-resistant breast cancer and triple-negative breast cancer

FTY720 has several advantages over available HDAC inhibitors as potential treatments for breast cancer patients: it is an orally bio-available pro-drug; it has already been approved for human use; it regulates expression of only a limited number of genes (a majority related to cholesterol and sphingolipid metabolism) compared to other HDAC inhibitors; it has good pharmacokinetics and a long half life; it suppresses several survival and proliferative pathways; and it is much less toxic, accumulates in tumor tissues, and both the phosphorylated and unphosphorylated forms target important pathways in breast cancer. Hence, we hope that our studies will pave the way for exploration of new clinical trials using FTY720 as a prototype of new adjuvant treatment strategies for hormonal resistant breast cancer. This might be particularly relevant in view of the increase in obesity that is now endemic.

4.2. The Impact on Other Disciplines

Although this work may not have a direct impact on other disciplines it might contribute to them, particularly in the treatment of cognitive impairment. HDAC inhibitors have shown promise as a treatment to combat the cognitive decline associated with aging and neurodegenerative disease, as well as to ameliorate the symptoms of depression and posttraumatic stress disorder, among others. Due to its unique features described above and its high brain penetration, FTY720 might be more effective than other HDAC inhibitors as an adjuvant therapy for erasing aversive memories (33).

4.3. The Impact on Technology Transfer

Nothing to report

4.4. The impact on Society Beyond Science and Technology

Nothing to report

5. CHANGES/PROBLEMS

Nothing to report.

6. PRODUCTS

Publications

1. Nitai C. Hait, Dorit Avni, Akimitsu Yamada, Masayuki Nagahashi, Tomoyoshi Aoyagi, Hiroaki Aoki, Catherine I. Dumur, Zara Zelenko, Emily J. Gallagher, Derek Leroith, Sheldon Milstien1, Kazuaki Takabe and **Sarah Spiegel**. The Phosphorylated Prodrug FTY720 Is a Histone Deacetylase Inhibitor that Reactivates ERα Expression and Enhances Hormonal Therapy for Breast Cancer. Oncogenesis (2015) 4, e156; doi:10.1038/oncsis.2015.16

2. Melissa Maczis, Sheldon Milstien, and **Sarah Spiegel**. Sphingosine-1-Phosphate and Estrogen Signaling in Breast Cancer. J. Biol. Regulation (2015) In press.

Abstracts

*Nitai C. Hait, Dorit Avni, Akimitsu Yamada, Masayuki Nagahashi, Tomoyoshi Aoyagi, Sheldon Milstien, Kazuaki Takabe and **Sarah Spiegel**. FTY720 Administration Reduces Breast Cancer Progression. Southeastern Regional Lipid Conference (SERLC) 49th Annual Conference. November 5-7, 2015. Cashiers, NC.

*Nitai C. Hait, Dorit Avni, Akimitsu Yamada, Masayuki Nagahashi, Tomoyoshi Aoyagi, Sheldon Milstien, Kazuaki Takabe and **Sarah Spiegel.** FTY720-P Is a Potent Inhibitor of Class I Histone Deacetylases that Enhances Histone Acetylation, Reactivates ERα Expression, and Increases Hormonal Therapeutic Sensitivity of Breast Cancer. AACR abstract, April 18-22, 2015, Philadelphia, PA.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals that have worked on the project

Name: Sarah Spiegel
Project Role: PI– No change

Name: Sheldon Milstien
Project Role: Co-Investigator – No change

Name: Kazuaki Takabe
Project Role: Co-Investigator – No change

Name: Clement Oyeniran
Project Role: Postdoctoral Fellow – No change
If his performance does not improve, we intend to replace him.

Name: Nita Hait
Project Role: Co-investigator
Researcher Identifier (e.g. ORCID ID): 0000-0002-9433-5498
Nearest person month worked: 7
Contribution to Project: Dr. Hait discovered that FTY720-P is an HDAC inhibitor and has performed the work with the transgenic mice.

Name: Melissa Maczis
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID):0000-0002-8610-2475
Nearest person month worked: 2
Contribution to Project: Ms. Maczis has recently joined the lab as a graduate student. She is being trained to carry out experiments with breast cancer models.
Funding Support: Ms. Maczis is supported by the VCU School of Medicine graduate program.

Name: Andreia Leopoldino
Project Role: Visiting Scientist
Researcher Identifier (e.g. ORCID ID):0000-0002-8313-4754

Nearest person month worked: 2
Contribution to Project: Dr. Leopoldino is a visiting scientist from Brazil. She came to my lab to gain knowledge in sphingolipid metabolites signaling in inflammation and cancer and is especially interested in the use of FTY720 for cancer treatment.
Funding Support: Dr. Leopoldino has a scholarship from Brazil.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No

What other organizations were involved as partners?
Nothing to Report

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9. **APPENDICES**

1. Nitai C. Hait, Dorit Avni, Akimitsu Yamada, Masayuki Nagahashi, Tomoyoshi Aoyagi, Hiroaki Aoki, Catherine I. Dumur, Zara Zelenko, Emily J. Gallagher, Derek Leroith, Sheldon Milstien1, Kazuaki Takabe and **Sarah Spiegel**. The Phosphorylated Prodrug FTY720 Is a Histone Deacetylase Inhibitor that Reactivates ERα Expression and Enhances Hormonal Therapy for Breast Cancer. Oncogenesis (2015) 4, e156; doi:10.1038/oncsis.2015.16
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ORIGINAL ARTICLE

The phosphorylated prodrug FTY720 is a histone deacetylase inhibitor that reactivates ERα expression and enhances hormonal therapy for breast cancer

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Estrogen receptor-α (ERα)-negative breast cancer is clinically aggressive and does not respond to conventional hormonal therapies. Strategies that lead to re-expression of ERα could sensitize ERα-negative breast cancers to selective ER modulators. FTY720 (fingolimod, Gilenya), a sphingosine analog, is the Food and Drug Administration (FDA)-approved prodrug for treatment of multiple sclerosis that also has anticancer actions that are not yet well understood. We found that FTY720 is phosphorylated in breast cancer cells by nuclear sphingosine kinase 2 and accumulates there. Nuclear FTY720-P is a potent inhibitor of class I histone deacetylases (HDACs) that enhances histone acetylations and regulates expression of a restricted set of genes independently of its known effects on canonical signaling through sphingosine-1-phosphate receptors. High-fat diet (HFD) and obesity, which is now endemic, increase breast cancer risk and have been associated with worse prognosis. HFD accelerated the onset of tumors with more advanced lesions and increased triple-negative spontaneous breast tumors and HDAC activity in MMTV-PyMT transgenic mice. Oral administration of clinically relevant doses of FTY720 suppressed development, progression and aggressiveness of spontaneous breast tumors in these mice, reduced HDAC activity and strikingly reversed HFD-induced loss of estrogen and progesterone receptors in advanced carcinoma. In ERα-negative human and murine breast cancer cells, FTY720 reactivated expression of silenced ERα and sensitized them to tamoxifen. Moreover, treatment with FTY720 also re-expressed ERα and increased therapeutic sensitivity of ERα-negative syngeneic breast tumors to tamoxifen *in vivo* more potently than a known HDAC inhibitor. Our work suggests that a multipronged attack with FTY720 is a novel combination approach for effective treatment of both conventional hormonal therapy-resistant breast cancer and triple-negative breast cancer.

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INTRODUCTION

The majority of breast tumors express the estrogen receptor-α (ERα) that plays important roles in breast cancer pathogenesis and progression, and hormonal therapies such as tamoxifen (TAM) are the first line of adjuvant therapy.[1,2] Unfortunately, 30% of these patients will ultimately fail therapy because of *de novo* or acquired resistance. Moreover, patients with ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (also known as ErbB-2) triple-negative breast cancer, which is aggressive with high recurrence, metastatic and mortality rates,[3] do not respond to hormonal therapies and have limited treatment options. Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis[4] that may be due in part to the high frequency of triple-negative breast cancer and ineffectual hormonal therapy.[5] As hormonal therapy is so effective with relatively few side effects, the possibility of reversing hormonal unresponsiveness is an appealing treatment approach. Histone deacetylases (HDACs) are negative regulators of ERα transcription, and HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA, vorinostat) and trichostatin A, have been shown to reactivate ERα expression in ERα – negative breast cancer cells and reverse TAM resistance in preclinical studies.[6–8] Encouragingly, in phase II clinical trials, the combination of vorinostat and TAM showed promising activity in reversing hormone resistance.[9]

FTY720 (fingolimod), the Food and Drug Administration (FDA) approved prodrug for the treatment of multiple sclerosis, is phosphorylated *in vivo* by sphingosine kinase 2 (SphK2) to its active form FTY720-phosphate (FTY720-P), a mimetic of sphingosine-1-phosphate (S1P) and an agonist of four S1P receptors (S1PRs) that interferes with immune cell trafficking by inducing internalization and degradation of S1PR1.[10] However, FTY720 has strong anticancer effects *in vitro* and *in vivo* in various types of cancers including breast[11,12] that are not well understood independently of its effects on immune cell trafficking.[13] Although some of its actions have been attributed to FTY720-P acting as a functional antagonist of S1PR1, reducing persistent activation of the transcription factor STAT3 (signal transducer and activator of transcription 3) important in malignant progression,[14–16] others have shown that the unphosphorylated FTY720 is an activator of protein phosphatase 2A, a tumor suppressor that is inactivated in many cancers.[17,18] However, our recent study suggests that

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FTY720-P and not FTY720 binds and inhibits recombinant class I HDACs.[19] Because it is generally believed that FTY720 in cancer cells is phosphorylated at the plasma membrane by SphK2 to form FTY720-P that acts via S1PRs, we asked where FTY720 is phosphorylated in breast cancer cells and whether FTY720-P also inhibits HDACs in these cells and in tumors to regulate histone acetylation and gene expression, and can be used to re-express ERα in ER-negative aggressive breast carcinoma for hormonal therapies.

RESULTS

SphK2 produces FTY720-P in the nucleus of breast cancer cells that inhibits class I HDACs

Following treatment with FTY720, an analog of sphingosine, FTY720-P is produced and accumulates over time in the nucleus of human and murine breast cancer cells in agreement with the predominant nuclear localization of SphK2 in these cells (Figures 1a, c, and f). Nuclear S1P levels were concomitantly decreased by almost twofold in these cell lines after FTY720 treatment because of decreased phosphorylation of the endogenous substrate sphingosine (Figures 1a and c). Interestingly, although it is generally assumed that most of the actions of the phosphorylated active form of FTY720 are at the plasma membrane to modulate S1PR signaling,[10] much more FTY720-P was present in cells than secreted into the media where it can interact with S1PRs (Figures 1b and d). Overexpression of SphK2 robustly increased the formation of nuclear FTY720-P by >20-fold in MDA-MB-231 cells (Figure 1e) and 100-fold in MCF7 cells (Figure 1g), whereas catalytically inactive SphK2^{G212E} had no significant effect on phosphorylation of FTY720 or formation of nuclear S1P. In agreement with our previous results showing that FTY720-P inhibits recombinant class I HDACs,[19] we observed that FTY720-P but not FTY720 itself inhibited endogenous class I HDACs (HDAC1–3 and HDAC8) immunoprecipitated from nuclear extracts with the corresponding antibodies as potently as the pan HDAC inhibitor SAHA (Supplementary Figure S1). However, in contrast to SAHA that inhibited class II HDAC7, FTY720-P did not.

Phosphorylated FTY720 enhances histone acetylations and regulates gene expression independently of S1PRs

Because the majority of FTY720-P is produced in the nucleus of breast cancer cells where its target HDACs are located, we next examined its effects on histone acetylation. Concomitant with

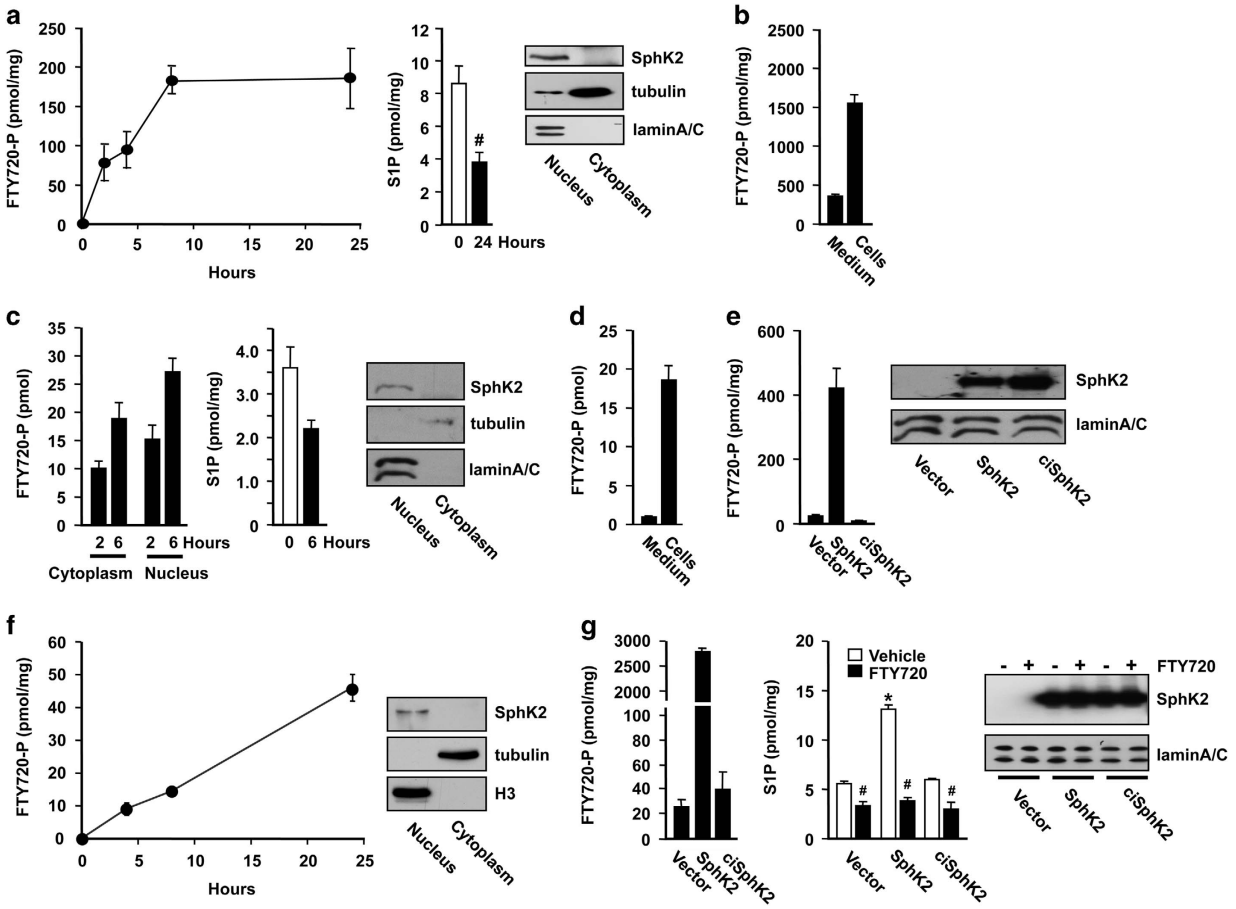


Figure 1. FTY720-P is produced in the nucleus of breast cancer cells by SphK2. Breast cancer cell lines, murine 4T1 (**a, b**), human MDA-MB-231 (**c, d**) and human MCF7 (**f**) were treated with 5 μM FTY720. (**a, c**) Nuclear levels of FTY720-P and S1P were determined by liquid chromatography, electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS) at the indicated times. Equal amounts of protein from nuclear and cytosolic fractions were analyzed by immunoblotting with SphK2 antibody. Antibodies against histone H3 or laminA/C and tubulin were used as nuclear and cytosol markers. (**b**, **d**) Total intracellular and secreted FTY720-P were determined in 4T1 cells after 8 h and MDA-MB-231 cells after 6 h of FTY720 treatment, respectively. MDA-MB-231 cells (**e**) and MCF7 cells (**g**) transfected with vector, SphK2 or catalytically inactive SphK2^{G212E} (ciSphK2) were treated with vehicle or 5 μM FTY720 for 6 and 24 h, respectively. Nuclear levels of FTY720-P and S1P were determined by LC-ESI-MS/MS. Data are mean ± s.d. $^{*}P < 0.005$ compared with vector; $^{\#}P < 0.005$ compared with vehicle. Equal expression of nuclear SphK2 was confirmed by immunoblotting.

increased nuclear FTY720-P (Figure 1), FTY720 increased acetylation of specific lysines of histone H3, H4 and H2B in MCF7 and 4T1 cells (Figures 2a and b). Similar results were found with MDA-MB-231 cells (Supplementary Figure S2a). In order to demonstrate that these effects are because of the intranuclear action of FTY720-P, experiments were carried out with purified MCF7 nuclei devoid of S1PRs. FTY720-P more potently than S1P enhanced histone acetylation in these nuclei (Figure 2c). Treatment of MCF7 cells with the SphK2 inhibitor K145 reduced FTY720-mediated histone acetylation (Supplementary Figure S2b). Importantly, addition of FTY720 itself also increased histone acetylation in these nuclei in a SphK2-dependent manner, as this effect was further enhanced by overexpression of SphK2 (but not catalytically inactive SphK2^{G212E}) (Figure 2d) and was prevented by its downregulation (Figure 2f). These effects correlated with the extent of formation of FTY720-P (Figures 2e and g).

To conclusively demonstrate that the effects on histone acetylation are due to direct intranuclear action of FTY720-P on HDAC activity independently of canonical signaling through S1PRs, cells were treated with FTY720-P that activates all S1PRs except S1PR2.[20] Although treatment of cells with FTY720-P, as expected, increased S1PR-mediated phosphorylation of ERK1/2, it did not induce significant changes in histone acetylation or in HDAC activity, in contrast to the significant effects of treatment with FTY720 or SAHA (Figures 2h and i).

Microarray analysis also indicated that the effects of FTY720 on gene expression in breast cancer cells are clearly distinguished from S1P receptor occupancy (Figure 3a). Unsupervised cluster analysis on 22 277 probe sets as well as supervised hierarchical cluster analyses demonstrated that there were no major differences in the clustering of the gene expression profiles between the naive, vehicle-treated and S1P-treated groups, whereas significant differences were observed in gene clustering between them and the FTY720- and SAHA-treated groups (Figure 3a). Although activation of S1PRs did not significantly alter gene expression, FTY720 treatment significantly changed the profiles of 713 genes. In comparison, SAHA significantly altered expression of 3166 genes, of which 276 were common to FTY720 (Figure 3b). The gene ontology analysis revealed that the majority of the commonly affected genes were related to transcription followed by lipid and steroid biosynthesis, transport and metabolic processes (Figure 3c). In addition, regulation of cell growth and angiogenesis genes was also prominent. Together, these results indicate that breast cancer cells take up FTY720 and that FTY720-P produced in the nucleus by SphK2 inhibits class I HDACs and increases specific histone acetylations and regulates expression of a restricted subset of gene programs independently of S1PRs.

FTY720 treatment suppresses development and progression of spontaneous breast tumors in HFD-fed MMTV-PyMT transgenic mice

Aberrant expression of class I HDACs and dysregulation of global histone acetylations has been found in a many cancers, including breast, and HDACs are promising targets in cancer therapeutics

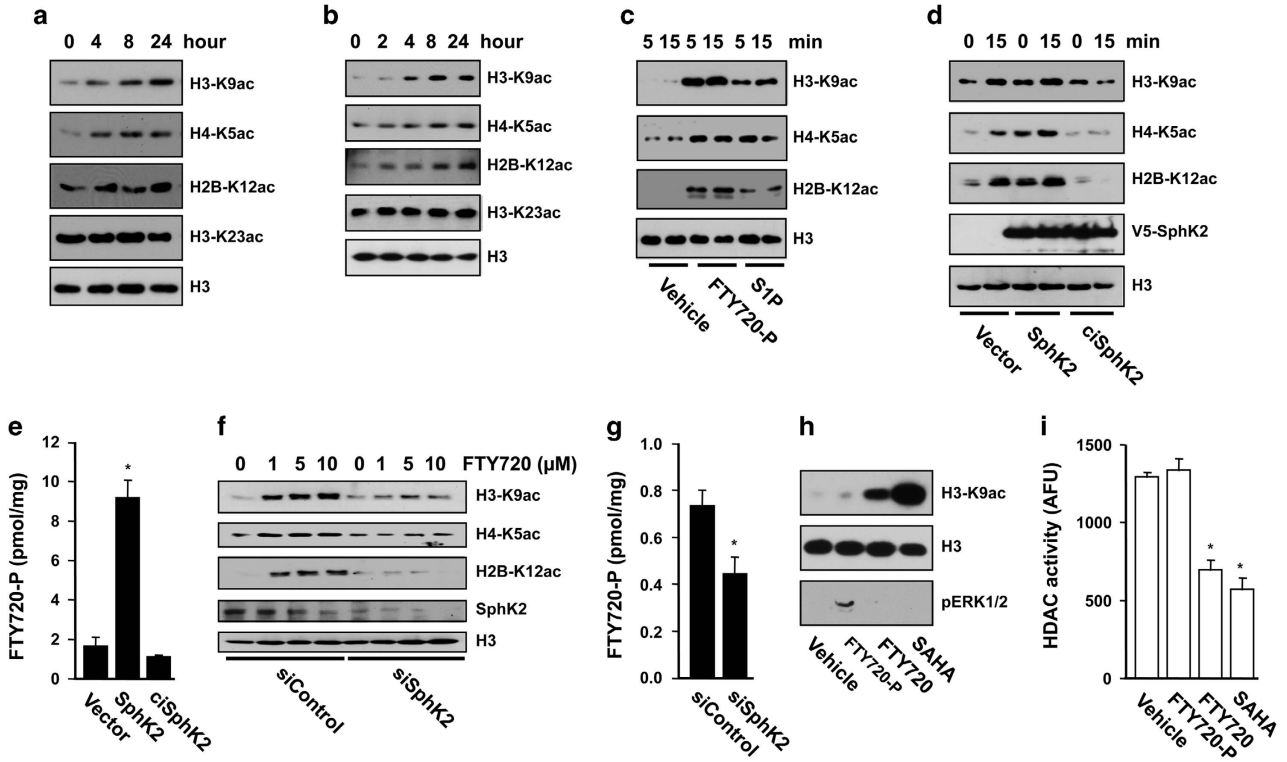
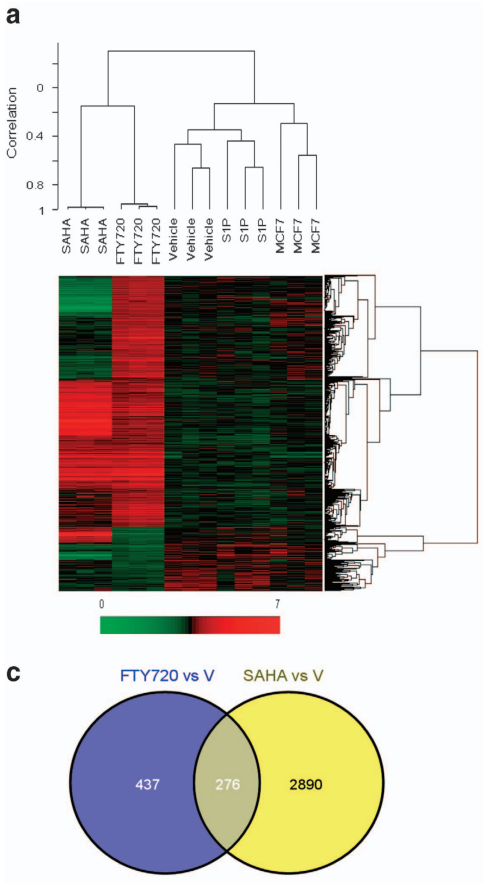


Figure 2. Nuclear FTY720-P enhances specific histone acetylations in breast cancer cells. MCF7 cells (**a**) and 4T1 cells (**b**) were treated with FTY720 (5 μM) for the indicated times. Histone acetylations in nuclear extracts were detected by immunoblotting with antibodies to specific histone acetylation sites. (**c**) Purified nuclei from naive MCF7 cells were incubated for the indicated times with vehicle, S1P (1 μM) or FTY720-P (1 μM) and histone acetylations determined. (**d**, **e**) Purified nuclei were isolated from MCF7 cells transfected with vector, SphK2 or ciSphK2 and treated with FTY720 (1 μM) for 15 min. (**f**, **g**) Purified nuclei were isolated from MCF7 cells transfected with siControl or siSphK2 and incubated with the indicated concentrations of FTY720 for 15 min. Histone acetylations were determined by immunoblotting (**d**, **f**) and levels of FTY720-P by liquid chromatography, electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS) (**e**, **g**). *$P < 0.05$. (**h, i**) Naive MCF7 cells were treated with vehicle, FTY720-P (100 nM), FTY720 (1 μM) or SAHA (2 μM) for 2 h, nuclear extracts were analyzed by western blotting with the indicated antibodies (**h**) and HDAC activity measured and expressed as arbitrary fluorescence units (AFU) (**i**). *$P < 0.001$.



b

GO Biological Process (Proportion on the 276 common probe sets)	n	Proportion
Unknown	51	18.48%
transcription	29	10.51%
lipid and steroid metabolic processes	27	9.78%
transport	11	3.99%
negative regulation of transcription from RNA polymerase II promoter	8	2.90%
metabolic process	7	2.54%
angiogenesis	6	2.17%
protein amino acid phosphorylation	5	1.81%
regulation of cell growth	5	1.81%
regulation of cyclin-dependent protein kinase activity	5	1.81%
cellular aromatic compound metabolic process	4	1.45%
cellular component movement	4	1.45%
response to hypoxia	4	1.45%
apoptosis	3	1.09%
autophagy	3	1.09%
citrate metabolic process	3	1.09%
endocytosis	3	1.09%
mRNA processing	3	1.09%
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3	1.09%
protein complex assembly	3	1.09%
protein folding	3	1.09%
regulation of transcription, DNA-dependent	3	1.09%
regulation of translation	3	1.09%
skeletal system development	3	1.09%
carbohydrate metabolic process	2	0.72%
cell cycle	2	0.72%
cell cycle checkpoint	2	0.72%
cytoskeleton organization	2	0.72%
DNA damage checkpoint	2	0.72%
DNA repair	2	0.72%
liver development	2	0.72%
MAPKKK cascade	2	0.72%
nucleosome assembly	2	0.72%
protein targeting to Golgi	2	0.72%
proteolysis	2	0.72%
ribosome biogenesis	2	0.72%
signal transduction	2	0.72%
somatic cell DNA recombination	2	0.72%
de novo pyrimidine base biosynthetic process	1	0.36%
activation of MAPK activity	1	0.36%

Figure 3. Microarray analysis of genes regulated by FTY720 and SAHA. Gene expression in naive MCF7 cells or MCF7 cells treated with vehicle, S1P (100 nM), FTY720 (1 μM) or SAHA (1 μM) for 24 h was determined by microarray analyses. (**a**) Heatmap showing supervised hierarchical clustering of 713 genes differentially expressed in FTY720-treated cells compared with naive. Expression level of a given gene is indicated by red (high) and green (low). Note that not all of the genes differentially regulated by SAHA are shown. (**b**) Venn diagram of genes differentially regulated by FTY720 and SAHA. (**c**) The gene ontology (GO) Biological Process analyses of 276 common probe sets regulated by SAHA and FTY720 treatment ranked for biological processes.

(reviewed in refs 21–24). Therefore, it was of interest to examine whether the HDAC inhibitory activity of FTY720-P could mitigate breast cancer development in a mouse model. Because diet, particularly fat intake, contributes to the development and progression of breast cancer and has been associated with worse prognosis,[4,5,25] MMTV-PyMT transgenic mice, which spontaneously develop breast cancer that closely mimics progression of the human disease,[26,27] were fed a high-fat diet (HFD). Female PyMT transgenic mice on a normal chow diet spontaneously developed palpable mammary tumors by 7.5 weeks (Figure 4a). In agreement with others,[28,29] feeding a HFD accelerated the onset of tumors that were palpable by 6 weeks (Figure 4a) and increased tumor multiplicity and size (Figures 4a and b). Although FTY720 administration decreased tumor burden without affecting onset in mice on normal diet (Figure 4a), it significantly increased the latency for appearance of palpable tumors to 7.5 weeks and dramatically suppressed tumor development in mice on HFD (Figures 4a–c). In addition, HFD-fed PyMT mice exhibited more advanced mammary carcinogenic lesions with poorly differentiated malignant cells of dissimilar cell shape and size (Figure 4d). These changes were all mitigated by FTY720 treatment. Consistent with the profound effect on tumor size, there was a significant increase in proliferation determined by Ki67 staining in the mice fed HFD compared with normal diet, which was decreased by FTY720 (Figures 4d and e). Conversely, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining revealed a large increase in apoptotic cells in tumors from FTY720-treated MMTV-PyMT transgenic mice (Figures 4d and e).

FTY720 treatment reverses HFD-induced HDAC activity and loss of estrogen and progesterone receptors in advanced carcinoma

In agreement with the advanced carcinoma observed in animals fed with HFD and consistent with a previous report,[29] expression of cyclin D1 was elevated (Figures 4d and 5a) and immunohistochemistry revealed high intensity of cyclin D1-positive clusters within these tumors (Figure 4d). In contrast, ERα protein and mRNA as well as PR mRNA were significantly reduced (Figure 5a and f), all of which are associated with poor prognosis in human breast cancers.[30] Notably, these characteristics of advanced tumorigenic mammary lesions were reversed by FTY720 administration to the HFD-fed transgenic mice (Figure 5b). Moreover, FTY720 treatment clearly induced nuclear ERα expression (Figure 5b, c, and f). Intriguingly, HFD reduced acetylation of H3-K9, H4-K5 and H2B-K12 in breast tumors that corresponded with increased nuclear HDAC activity (Figures 5a,d). Conversely, FTY720 administration dramatically increased these specific histone acetylations determined by immunoblotting (Figure 5b) and confirmed by immunohistochemical staining of H3-K9ac in mammary tumors (Figure 5c). FTY720 also reduced HDAC activity in breast tumors (Figure 5d), concomitant with marked production

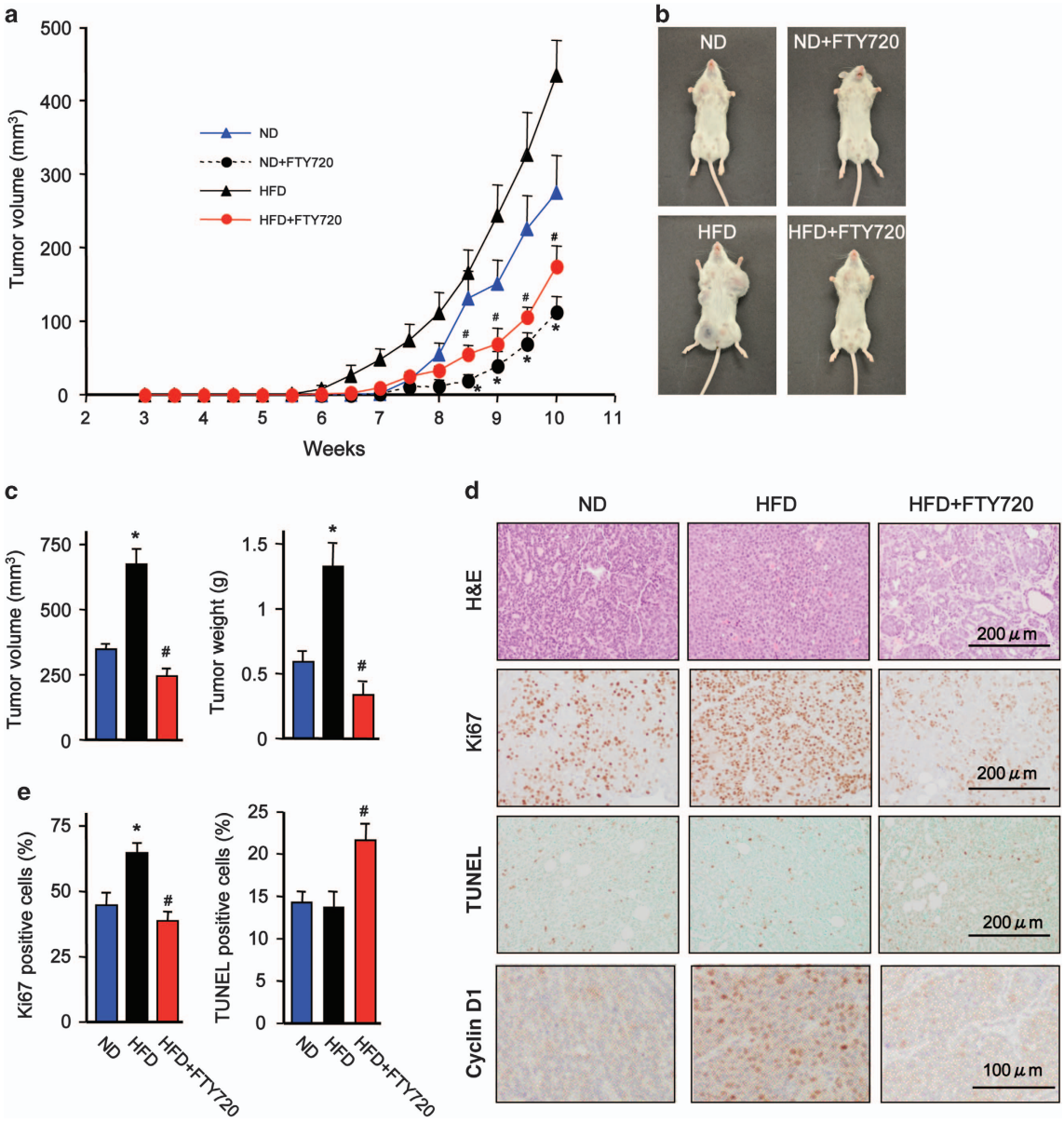


Figure 4. FTY720 treatment suppresses advanced tumorigenic mammary lesions in HFD-fed PyMT transgenic mice. Female PyMT transgenic mice were fed with a normal diet (ND) or a Western HFD, and were treated daily with saline or FTY720 (1 mg/kg) by gavage starting after weaning. (**a**) Tumor volumes were determined at the indicated times. (**b**) Representative images of 10-week-old female PyMTTg mice fed with ND or HFD without or with FTY720. Note the difference in the size of the tumors in the mammary pads. (**c**) Tumor volumes and weights were determined at 11 weeks. (**d**, **e**) Tumor sections were stained with hematoxylin and eosin (H&E), proliferation determined by Ki67 staining, apoptosis by TUNEL and cyclin D1 expression determined by immunohistochemistry. Scale bars: 200 μm and 100 μm, as indicated. (**e**) Quantification of Ki67- and TUNEL-positive cells. Data are mean ± s.e.m. $^{\#}P < 0.05$ compared with ND; $^{*}P < 0.05$ compared with HFD.

of nuclear FTY720-P compared with FTY720 (Figure 5e) and increased mRNA levels of ERα and PR, without affecting expression of ErbB2 (Figure 5f). Interestingly, in tumor-free mammary fat pads from MMTV-PyMT transgenic mice or from naive C57BL/6 mice, HFD reduced HDAC activity and increased histone acetylations (Supplementary Figure S3).

FTY720 sensitizes triple-negative breast cancer cells to TAM by reactivation of silenced ERα expression

HDACs are negative regulators of the ERα transcriptional complex and HDAC inhibitors have been shown to epigenetically restore ERα expression and reverse TAM resistance in hormone-resistant breast cancer cells[6,7,31,32] and in preclinical animal studies.[33,34] As we found that FTY720 is phosphorylated in the nucleus of ERα-negative MDA-MB-231 and 4T1 breast cancer cells by SphK2 (Figure 1) and that FTY720-P is a potent class I HDAC inhibitor, we asked whether it induces ERα re-expression in ERα-negative breast cancer. Although FTY720 alters expression of many fewer genes than SAHA (Figure 3c), FTY720 more potently than SAHA enhanced ERα expression in 4T1 cells (Figure 6a), whereas in MDA-MB-231 cells, it enhanced ERα mRNA and protein expression to the same extent as SAHA (Figures 6b and c). Similar to SAHA, FTY720 also enhanced expression of PR, one of the ERα target genes (Figures 6b and c). As expected, neither SAHA nor FTY720 had a significant effect on expression of ERβ (Figure 6b). Moreover,

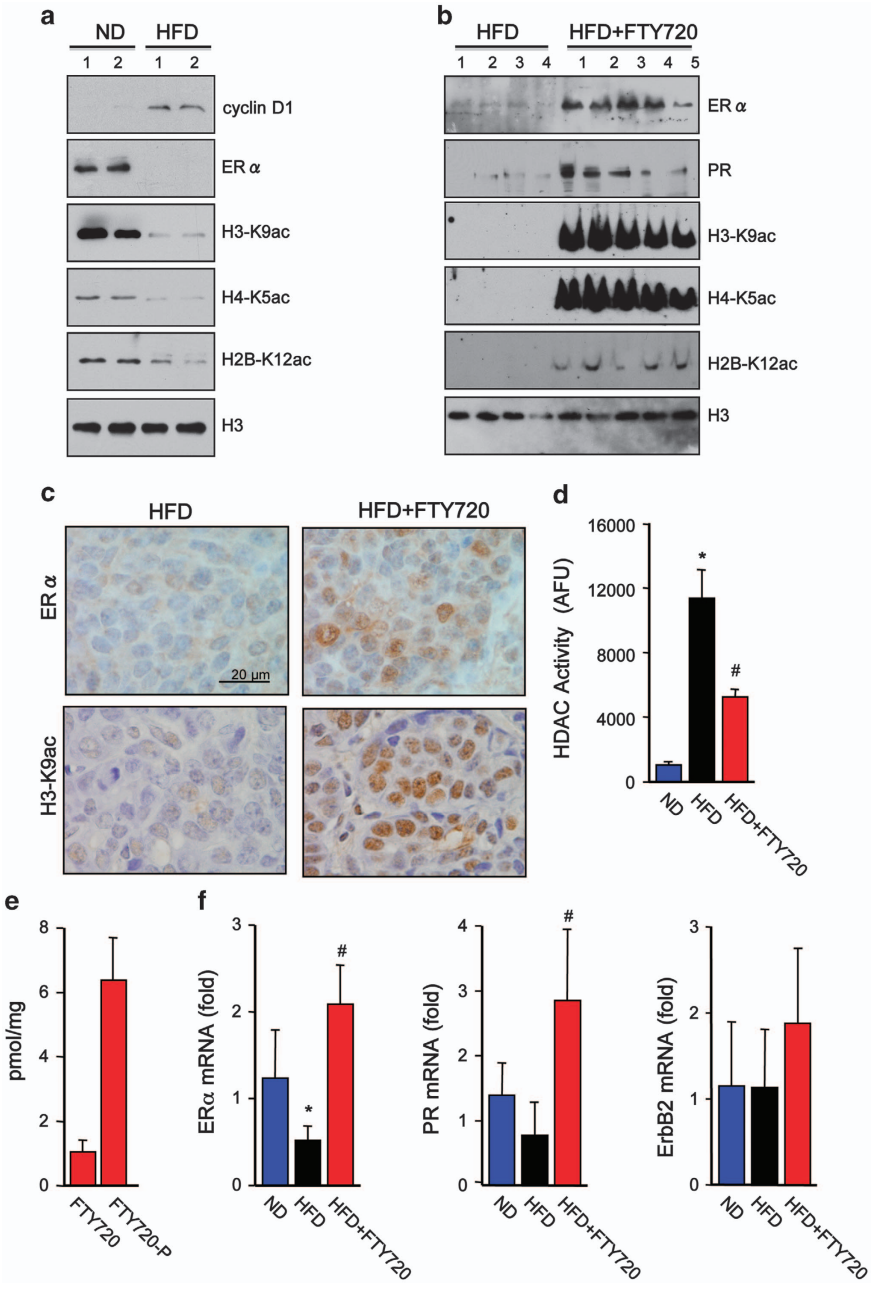


Figure 5. FTY720 treatment reverses HFD-induced loss of estrogen and progesterone receptors in PyMT transgenic mice. Female PyMT transgenic mice were fed with a normal diet (ND) or a Western HFD, and were treated daily with saline or FTY720 (1 mg/kg) by gavage starting after weaning ($n = 5$ each), as indicated. (**a**, **b**) Nuclear extracts from tumors were analyzed by western blotting with the indicated antibodies. (**c**) Representative images of tumor sections immunostained with anti-ERα or anti-H3-K9ac antibodies. Scale bars: 20 μm. (**d**) HDAC activity in nuclear extracts of tumors was determined and expressed as arbitrary fluorescence units. (**e**) FTY720 and FTY720-P levels in nuclear extracts of tumors from mice on HFD treated with FTY720 were measured by liquid chromatography, electrospray ionization/tandem mass spectrometry (LC-ESI-MS/MS). (**f**) ERα, PR and ErbB2 mRNA levels in tumors were quantified by quantitative real-time PCR (QPCR) and normalized to *Gapdh*. Data are mean ± s.e.m. $^{*}P < 0.05$ compared with ND; $^{\#}P < 0.05$ compared with HFD.

chromatin immunoprecipitation assays revealed that FTY720, even more potently than SAHA, enhanced association of acetylated histone H3 at the ERα promoter (Figure 6d), indicating that reactivation of ERα expression correlates with ERα promoter hyperacetylation. In agreement, treatment of these ERα-negative cells with E2 enhanced their proliferation only in the presence of FTY720 (Figure 6e). We next examined whether FTY720, which restores ERα expression in ERα-negative breast cancer cells, could also induce sensitivity to TAM, an ERα antagonist. As expected, TAM alone at concentrations up to 10 μM did not inhibit growth of MDA-MB-231 cells (Figure 6f). Like other HDAC inhibitors,[6,31,32] FTY720 reduced growth in a concentration-dependent manner and importantly sensitized the cells to TAM. For example, although a concentration of 2.5 μM TAM or FTY720 alone only reduced growth of MDA-MB-231 cells by 9.3% or 29.3%, respectively, when combined, cell growth was inhibited by >63% (Figure 6f), with a Synergistic Index of 0.23. Similarly, in highly metastatic ERα-negative 4T1 murine mammary carcinoma cells, FTY720 also greatly enhanced the growth inhibitory effect of TAM (Figure 6g), with a Synergistic Index of 0.23.

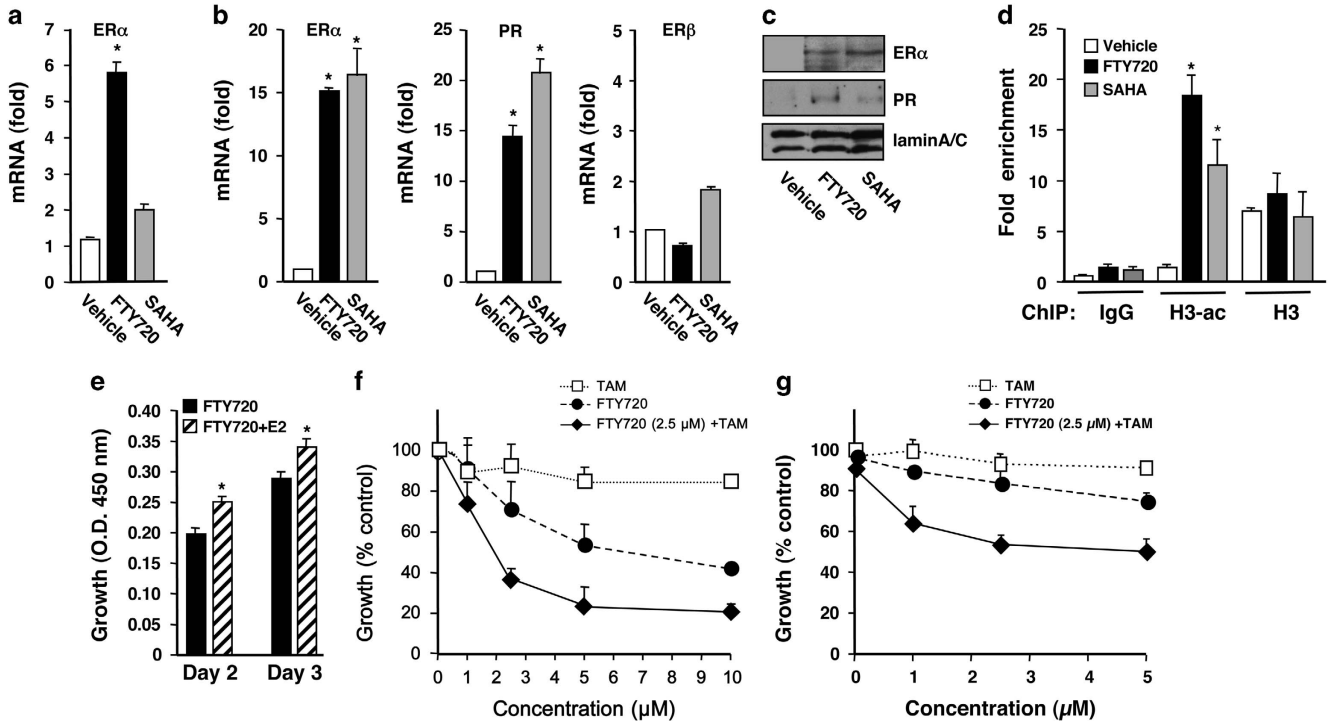


Figure 6. FTY720 induces ERα expression in ERα-negative human and murine breast cancer cells and sensitizes them to tamoxifen. 4T1 (**a**) and MDA-MB-231 (**b**) cells were treated with FTY720 (5 μM) or SAHA (1 μM) for 24 h. ERα, PR and ERβ mRNA levels were determined by quantitative real-time PCR (QPCR) and normalized to GAPDH. (**c**) Proteins in MDA-MB-231 nuclear extracts were analyzed by immunoblotting with the indicated antibodies. LaminA/C was used as a loading control. (**d**) MDA-MB-231 cells were subjected to chromatin immunoprecipitation (ChIP) analyses with antibodies to H3-ac, H3 or normal rabbit IgG, as indicated. The precipitated DNA was analyzed by real-time PCR with primers amplifying the core promoter sequence of the ERα gene. Relative binding to the promoter is expressed as fold enrichment compared with input. Data are mean ± s.d. $^*P < 0.003$ compared with vehicle. (**e**) MDA-MB-231 cells were treated with FTY720 (1 nM) without or with 10 nM E2 for the indicated days and cell proliferation was determined by WST assay. (**f**, **g**) MDA-MB-231 cells (**f**) or 4T1 cells (**g**) were treated with the indicated concentrations of TAM or FTY720, or with 2.5 μM FTY720 with increasing concentrations of TAM for 48 h and cell proliferation determined. Data are expressed as % of untreated control.

FTY720 increases therapeutic sensitivity of ERα-negative syngeneic breast tumors to TAM

As we have found that FTY720 treatment induces functional ERα reactivation and sensitizes ERα-negative breast cancer cells to TAM *in vitro*, we sought to determine whether FTY720 also enhances antiestrogen therapy *in vivo*. We utilized a syngeneic mouse metastatic breast cancer model instead of conventional xenografts in immunocompromised nude mice that more accurately mimics human breast cancer.[35,36] ERα-negative 4T1 cells were orthotopically implanted into the second mammary fat pad of immunocompetent mice and randomized to insure similar tumor burdens before treatment. 4T1 cells produced large primary tumors in the chest mammary fat pad that were not significantly reduced by TAM administration (Figure 7a). Orally administered FTY720 reduced tumor growth, an effect that was significantly potentiated by coadministration of TAM (Figures 7a and b). Strikingly, FTY720 enhanced the antitumor efficacy of TAM more than SAHA. TUNEL staining also revealed a large increase in apoptotic cells in tumors from FTY720- plus TAM-treated mice as compared with tumors from mice treated with each separately (Figure 7c). Immunohistochemical analysis revealed that nuclear expression of ERα was increased in tumors from FTY720-treated mice that was more prominent when combined with TAM than even in tumors from mice treated with the combination of SAHA and TAM (Figure 7d). Consistent with the decreased nuclear HDAC activity in tumors from animals treated with FTY720 or SAHA (Figure 7e), acetylation of histone H3-K9, H4-K5 and H2B-K12 was increased in these tumors (Figure 7g), leading to re-expression of ERα (Figures 7f and g). Taken together, these data suggest that FTY720 induces epigenetic ERα reactivation *in vivo* to enhance hormonal therapy of ERα-negative breast cancer.

DISCUSSION

Hormonal therapies, including selective estrogen receptor modulators and aromatase inhibitors, are the standards of care for treatment of ER-positive breast cancer. However, development of resistance to hormone therapies in advanced breast cancer is a major obstacle. Therefore, epigenetic reactivation of silenced ERα expression by HDAC inhibitors has emerged as an attractive potential mode of therapy for these breast cancer patients.[9] Moreover, treatment of triple-negative breast cancer, which has poor prognosis, remains challenging because the tumors are more aggressive and resistant to hormonal therapy.[30] Epigenetic modifications are responsible for the lack of ERα expression and HDACs 1, 2 and 3 are overexpressed in breast cancer and correlate with more aggressive tumor type.[37] In agreement, we found that consumption of HFD by MMTV-PyMT transgenic mice induced more aggressive, poorly differentiated tumors with increased HDAC activity. In contrast, in mammary pads from naive animals or those without tumors, HFD reduced HDAC activity, supporting the notion that HFD and obesity increase acetylations of histones with changes in the epigenome.[38] Similar to our findings, it was previously reported that HFD-induced HDAC activity plays important roles in epigenetic regulation of tumor suppressor genes involved in colorectal tumor growth and progression,

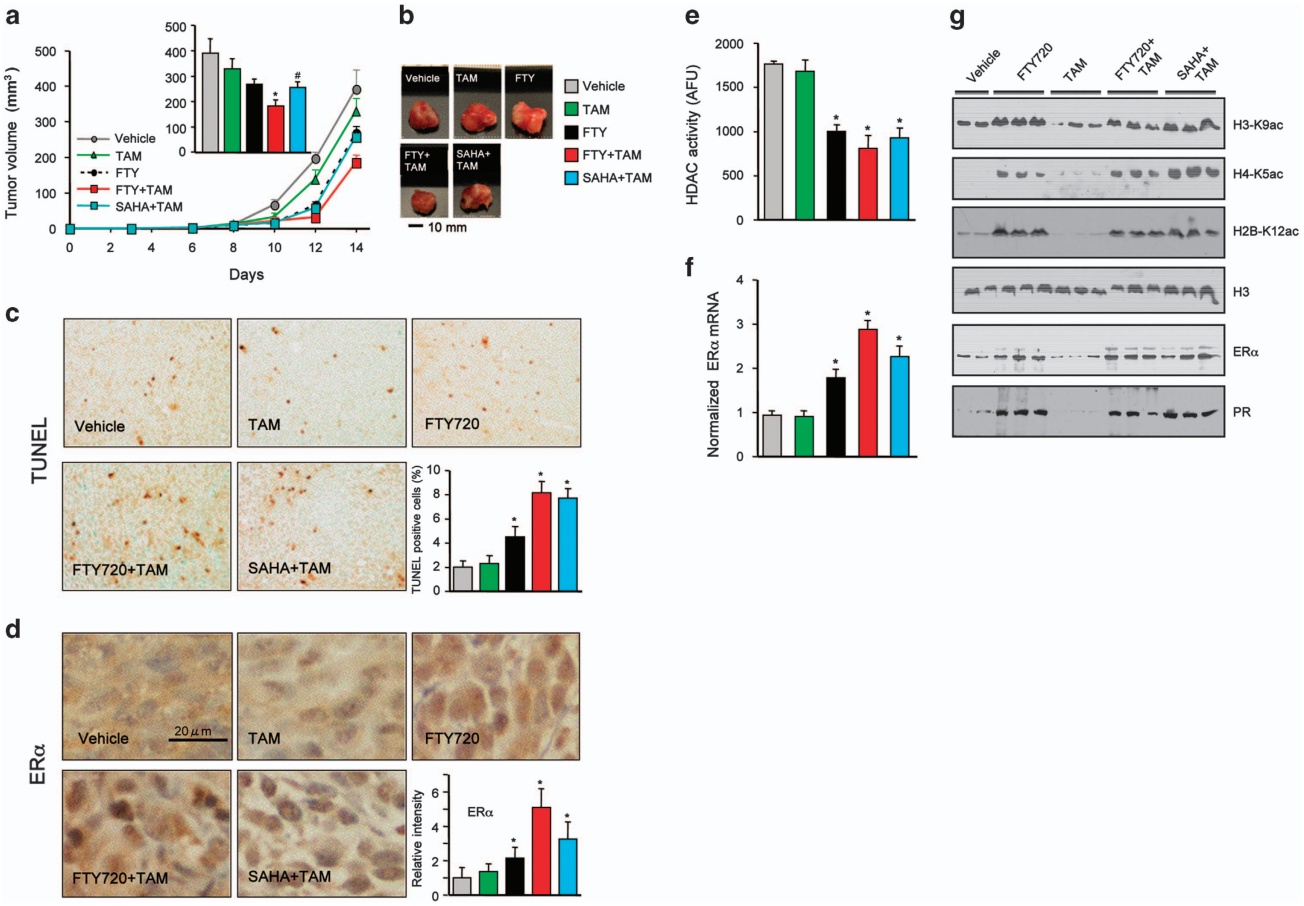


Figure 7. FTY720 reduces breast tumor growth and enhances anticancer effectiveness of TAM in ERα-negative 4T1 syngeneic xenografts. 4T1 cells were surgically implanted into the second mammary fat pads under direct vision. Tumor-bearing mice were randomized into five groups 2 days after implantation and then treated with vehicle, FTY720 (1 mg/kg), TAM (25 mg/kg), FTY720 plus TAM or SAHA (intraperitoneal (i.p.) 20 mg/kg) plus TAM by gavage daily till day 15 ($n = 8$). (**a**) Tumor volumes were measured daily. (Insert) Tumor volumes on day 15. (**b**) Representative tumors. $^{*}P < 0.01$, $^{\#}P < 0.05$ compared with vehicle. (**c**, **d**) Immunohistochemical staining of tumor sections for TUNEL (**c**) and ERα (**d**). Scale bar: 20 μm. Quantifications of TUNEL-positive cells and ERα intensity are shown. $^{*}P < 0.05$ compared with vehicle or TAM. (**e**) HDAC activity in nuclear extracts of tumors was determined and expressed as arbitrary fluorescence units. (**f**) Expression of ERα in the tumors was analyzed by quantitative real-time PCR (QPCR) and normalized to *Gapdh*. (**g**) Nuclear extract proteins were analyzed by western blotting with the indicated antibodies. Histone H3 was used as loading control. Data are mean ± s.e.m. $^{*}P < 0.01$ compared with vehicle or TAM.

including increased epithelial–mesenchymal transition and tumor inflammation in a xenograft model.[39]

Several HDAC inhibitors have been developed that restored the efficacy of hormonal therapy in preclinical models[33,34,40] and a few have advanced to clinical trials.[9,41] Combination of the HDAC inhibitor vorinostat with TAM for patients with ER-positive metastatic breast cancer progressing on hormonal therapy showed encouraging reversal of hormone therapy resistance.[9] Similar results were obtained in a phase II clinical trial in postmenopausal women with locally recurrent or metastatic ER-positive breast cancer progressing on treatment with a nonsteroidal aromatase inhibitor when combined with the HDAC inhibitor entinostat,[41] leading to a phase III clinical trial that is currently underway (Clinical-Trials.gov identifier: NCT02115282).

It was originally suggested that the prodrug FTY720 (fingolimod, Gilenya) approved for human use is phosphorylated at the plasma membrane by SphK2 to form FTY720-phosphate that acts via S1P receptors.[10,20] However, in this study we have demonstrated that FTY720 is predominantly phosphorylated to FTY720-P in the nucleus of both ER-positive and ER-negative breast cancer cells. Although it has been suggested that phosphorylation of FTY720 by SphK2 is a requirement for its induction of apoptosis *in vitro* and *in vivo*, the targets for this action have not been identified.[42] We are now showing that the active phosphorylated form of FTY720 is a potent class I HDAC inhibitor. This novel nuclear action of FTY720-P provides a new mechanism to explain the cytotoxic effects of FTY720 in cell culture and its preclinical antitumor efficacy in many xenograft and syngeneic cancer models.[43] Moreover, similar to other HDAC inhibitors, treatment with FTY720 enhances histone acetylation at the ERα promoter leading to its re-expression, and sensitizes ER-negative breast cancer cells to TAM therapy. Interestingly, even in HFD-fed PyMT transgenic mice that developed more advanced, poorly differentiated mammary tumors with increased HDAC activity and decreased expression of ERα and PR, oral administration of FTY720 not only suppressed development and progression of these spontaneous breast tumors, but also reduced HDAC activity in tumors and concomitantly induced expression of ERα and PR. Importantly, FTY720 treatment of breast tumor-bearing mice also induced re-expression of ERα in the tumor and greatly enhanced the anticancer efficacy of TAM, even more potently than a known HDAC inhibitor.

FTY720 has multiple beneficial anticancer activities. First, it has been convincingly shown that the unphosphorylated form is a potent activator of protein phosphatase 2A, a heterotrimeric serine/threonine phosphatase that counteracts the activity of many kinase-driven signaling pathways, including MEK and

AKT.[17,18] In this regard, reduced protein phosphatase 2A activity is a common event in breast cancer that could predict sensitivity to FTY720.[44] Second, unphosphorylated FTY720 also inhibits and induces proteasomal degradation of SphK1,[45] which is upregulated in breast cancer and correlates with poor prognosis and drug resistance.[46–49] High expression of SphK1 and S1PR1 are also associated with development of TAM resistance in ER-positive breast cancer patients.[50] Third, because FTY720-P is a functional antagonist of S1PR1, it can also suppress tumor growth by several S1PR1-dependent mechanisms. It was shown to decrease prosurvival/anti-apoptotic signaling from S1PR1 via suppression of proapoptotic Bim and upregulation of prosurvival Mcl-1 proteins.[51] Targeting S1PR1 with FTY720 also interferes with a major positive feedback loop for persistent STAT3 activation in breast tumor microenvironment critical for malignant progression.[14] Moreover, FTY720 by interfering with the upregulation of SphK1 and S1PR1 curtails the S1P/SphK1/S1PR1 feed-forward amplification loop that leads to nuclear factor-κB and persistent STAT3 activation that play important roles in the link between chronic inflammation and cancer.[16] Finally, in this paper we have uncovered a novel action of FTY720-P as a potent inhibitor of class I HDACs that acts similarly to other HDAC inhibitors to reactivate ERα expression and sensitize breast cancer cells to TAM therapy.

At first glance, the immunosuppressive action of FTY720 would seem to be an undesirable effect in cancer therapy. However, it has been shown that following treatment of tumor-bearing mice with FTY720, there was a significant reduction in accumulation of tumor-associated regulatory T cells and an increase in peripheral blood regulatory T cells, suggesting that FTY720 causes a block in blood-to-tumor regulatory T-cell recruitment that would allow more potent antitumor immunity.[52] Moreover, treatment of mice with FTY720 after tumors were established to block new T-cell trafficking from secondary lymphoid organs still enabled the increase in the capacity of tumor-infiltrating $CD8^+$ T cells to produce IL-2 and to proliferate and subsequent tumor rejection induced by combinatorial immunotherapy with anti-CTLA-4 and anti-PD-L1 monoclonal antibodies.[53]

As HDAC inhibitors are being developed for treatment of breast cancer acting through multiple epigenetic pathways, and numerous clinical trials are underway,[21–24] it is not surprising that FTY720 has such potent anticancer activity. FTY720 has several advantages over available HDAC inhibitors as potential treatments for breast cancer patients: it is an orally bioavailable prodrug; it has already been approved for human use; it regulates expression of only a limited number of genes (a majority related to cholesterol and sphingolipid metabolism) compared with other HDAC inhibitors; it has good pharmacokinetics and a long half-life; it suppresses several survival and proliferative pathways; it is much less toxic, accumulates in tumor tissues, and both the phosphorylated and unphosphorylated forms target important pathways in breast cancer. Hence, we hope that our studies will pave the way for exploration of new clinical trials using FTY720 as a prototype of new adjuvant treatment strategies for hormonal-resistant breast cancer.

MATERIALS AND METHODS

Cell culture and transfection

Human breast cancer cells MCF7 and MDA-MB-231 (ATCC, Manassas, VA, USA) and murine 4T1 breast cancer cells (Caliper Life Sciences, Waltham, MA, USA) were cultured and transfected with vector, SphK2 or catalytically inactive $SphK2^{G212E}$ as previously described.[54] SphK2 was downregulated by transfection with ON-TARGETplus SMARTpool siRNA against SphK2, and scrambled siRNA (Dharmacon, Lafayette, CO, USA) was used as control.[54] Cell growth was determined with WST-8 reagent and absorbance was measured at 450 nm.[55]

Nuclear extracts and immunoblotting

Nuclear extracts from tissues and cells were prepared and protein expression determined by immunoblotting as previously described.[54] Proteins were separated by SDS–polyacrylamide gel electrophoresis, transblotted to nitrocellulose and incubated with primary antibodies as indicated in figure legends, including rabbit polyclonal antibodies to: histone H3-K23ac (1:1000 dilution; EMD Millipore, Billerica, MA, USA); histone H3, H3-K9ac, H4-K5ac and H2B-K12ac (1:1000 dilution; Abcam, Cambridge, MA, USA); laminA/C, tubulin, p-ERK1/2, HDAC3 and HDAC7 (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA); HDAC1, HDAC2 and HDAC8 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); V5 (1:5000 dilution; Life Technologies, Grand Island, NY, USA); and SphK2 (1:1000 dilution).[54] Immunopositive bands were visualized by enhanced chemiluminescence using secondary antibodies conjugated with horseradish peroxidase (goat anti-rabbit or anti-mouse, 1:5000 dilution; Jackson ImmunoResearch, West Grove, PA, USA) and Super-Signal West Pico chemiluminescent substrate (Pierce Chemical Co., Rockford, IL, USA). Optical densities of bands associated with proteins of interest were quantified using AlphaEaseFC software (Alpha Innotech, Miami, FL, USA) and normalized to the optical densities of their respective tubulin bands.

Quantification of sphingolipids by mass spectrometry

Sphingolipids were measured by liquid chromatography, electro-spray ionization–tandem mass spectrometry (4000 QTRAP, AB Sciex, Framingham, MA, USA).[54]

HDAC activity measurements

Enzymatic activities of HDACs immunoprecipitated from nuclear extracts with specific antibodies were measured with fluorometric assay as previously described.[54].

Quantitative real-time PCR

Total RNA from cells or tumors was isolated with Trizol (life Technologies, Grand Island, NY, USA) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit and pre-mixed primer probe sets from Applied Biosystems (Foster City, CA, USA). Complementary DNA (cDNA) was amplified with the ABI 7900HT (Applied Biosystems).[54]

Chromatin immunoprecipitation

Cells were crosslinked with 1% formaldehyde for 10 min at 37 °C and then quenched with glycine, washed with cold phosphate-buffered saline, suspended in SDS buffer, sonicated and centrifuged. Supernatants were pre-cleared with protein G-Sepharose beads (GE Healthcare, Piscataway, NJ, USA) that were blocked with sonicated herring sperm DNA (Promega, San Louis Obispo, CA, USA) in IP buffer (16.7 mM Tris (pH 8), 16.7 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, containing 0.05 mg/ml bovine serum albumin). Chromatin was immunoprecipitated with rabbit poly-clonal anti-acetylated H3 or anti-H3 antibodies, or with control rabbit IgG.[54] DNA–protein complexes were pulled down with the protein G-Sepharose beads and then washed with low salt buffer, high salt buffer, LiCl buffer and Tris-EDTA buffer before eluting with 1% SDS in 0.1 M $NaHCO_3$. Crosslinks were reversed by heating at 65 °C overnight in 0.3 M NaCl, followed by proteinase K digestion for 1 h at 55 °C. Input samples were also similarly treated. DNA was purified with QIAquick PCR Purification Kit (Qiagen, Germantown, MD, USA). The ER promoter was analyzed by quantitative real-time PCR using SYBR Green Master Mix (Applied Biosystems) and the following primers: sense, 5′-GAACCGTCCGCAGCT CAAGATC-3′; anti-sense, 5′-GTCTGACCGTAGACCTGCGCGTTG-3′. Results were analyzed relative to input using the ΔCT method. Specific endogenous chromatin immunoprecipitation enrichments were all at least threefold greater than control.

Animal studies

All animal studies were conducted in the Animal Research Core Facility at VCU School of Medicine in accordance with the institutional guidelines. Animals were bred and maintained in a pathogen-free environment and all procedures were approved by the VCU Institutional Animal Care and Use Committee that is accredited by the Association for Assessment and Accreditation of laboratory Animal Care. All mice were kept on a 12-h light/dark cycle with free access to food.

Male MMTV-PyMT mice on a FVB/N background (Jackson Laboratories, Bar Harbor, MD, USA) were randomly bred with normal FVB/N females to obtain females heterozygous for the PyMT oncogene. Mice were fed a normal diet or HFD (TD.88137; Harlan Laboratories, Indianapolis, IN, USA) containing cholesterol (0.2%), total fat (21% by weight; 42% kcal from fat), saturated fatty acids (>60% of total fatty acids), sucrose (34% by weight), protein (17.3% by weight) and carbohydrate (48.5% by weight). Palpable mammary tumors developed as early as 6 weeks of age. Tumor size was measured with calipers every 3 days and total tumor volume was estimated by the cylinder formula.

For the syngeneic breast cancer model, 4T1 mouse mammary cancer cells were surgically implanted in the upper fat pads of female BALB/c mice (8 to 12 weeks of age, Jackson Laboratories) under direct vision as described previously.[35,36] Tumor-bearing mice were randomized 2 days after implantation into five treatment groups: vehicle, FTY720 (p.o. 1 mg/kg, Cayman Chemical Company, Ann Arbor, MI, USA), TAM (intraperitoneal 25 mg/kg; Sigma-Aldrich, St Louis, MO, USA), FTY720 plus TAM, and SAHA (intraperitoneal 20 mg/kg; Sigma-Aldrich) plus TAM. Tumors were measured regularly and tumor volumes calculated. At the indicated times, animals were killed by exsanguination, blood was collected, tumors excised, weighed, fixed in 10% neutral buffered formalin and embedded in paraffin or frozen in liquid nitrogen for morphological and immunofluorescence analyses.

Histopathological analysis

Tissue slices (5 μm) were stained with hematoxylin and eosin for morphological analysis. Frozen tissue samples were embedded in Optimal Cutting Medium (OCT 4583; Sakura Finetek, Torrance, CA, USA) for immunofluorescence analysis. Paraffin-embedded slides were deparaffinized, and antigen unmasking was carried out by microwave heating in citrate buffer for 20 min. Slides were incubated with 3% H_2O_2 and then with goat or horse serum (DAKO, Carpinteria, CA, USA) for 30 min at room temperature. After washing with phosphate-buffered saline, slides were incubated at 4 °C overnight with the following primary antibodies: ERα (Santa Cruz), H3-K9ac (Abcam), cyclin D1 (Cell Signaling) and Ki67 (Dako). Biotinylated secondary antibodies were added and incubated at room temperature for 20 min. After 5 min with streptavidin-HRP, sections were stained with DAB substrate and counterstained with hematoxylin. Slides were examined with a Zeiss Axioimager A1 (Jena, Germany) and images captured with an AxioCam MRc camera.

Gene expression microarrays

Total RNA was extracted using the MagMAX-96 for Microarrays Total RNA Isolation Kit (Life Technologies) in an automated manner using the MagMAX Express magnetic particle processor. RNA purity integrity was assessed by spectrophotometry at 260, 270 and 280 nm and by RNA 6000 Nano LabChips with the 2100 Bioanalyzer (Agilent Technologies, Carpenteria, CA, USA).[56] Single-strand cDNA was synthesized from 500 ng total RNA primed with a T7-(dT24) oligonucleotide. Second-strand cDNA synthesis was performed with *Escherichia coli* DNA Polymerase I, and cRNA biotinylated by *in vitro* transcription using the GeneChip 3' *in vitro* transcription Express Kit (Affymetrix, Santa Clara, CA, USA). After incubation at 37 °C for 16 h, labeled cRNA was purified using the GeneChip Sample Cleanup Module. Fragmented cRNA (10 μg) was hybridized on the GeneChip HG-U133A 2.0 array for 16 h at 60 r.p.m. in a 45 °C hybridization oven. Arrays were washed and stained with streptavidin phycoerythrin (Life Technologies) in the Affymetrix fluidics workstation. Every chip was scanned at a high resolution on the Affymetrix GeneChip Scanner 3000 7G and raw intensities for every probe were stored in electronic files by the GeneChip Operating Software v1.4.[36] Overall quality of each array was assessed by monitoring the 3′/5′ ratios for the housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), and the percentage of 'Present' genes (%P). Arrays exhibiting *GAPDH* 3′/5′ < 3.0 and %$P > 40$% were considered good-quality arrays. For microarray data analyses, background correction, normalization and estimation of probe set expression summaries and filtering and hierarchical cluster analyses were performed using the log-scale Robust Multi-array Analysis method[57] and BRB-ArrayTools v3.1.0 (NCI, Bethesda, MD, USA), respectively. Differentially expressed genes among the classes were identified by *t*-test analyses. To adjust for multiple hypotheses testing, the resulting *P*-values were used to obtain the false discovery rates using the *q*-value method. All analyses were performed on the R environment using functions provided by the BioConductor packages.[58]

Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student's *t*-test for comparison of two groups. $P < 0.05$ was considered significant. Experiments were repeated at least three times with consistent results. For animal studies, measurements were blind with respect to group assignments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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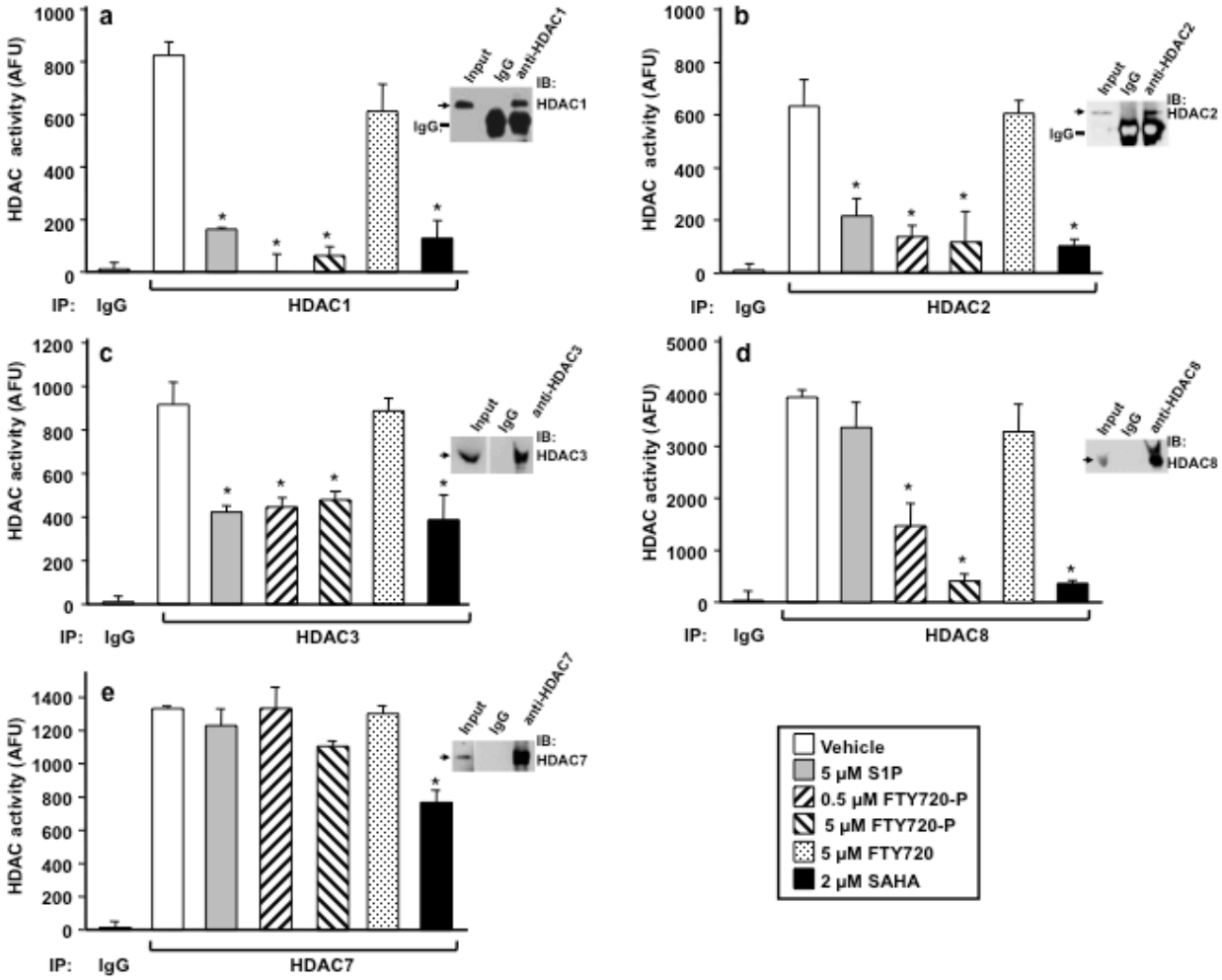
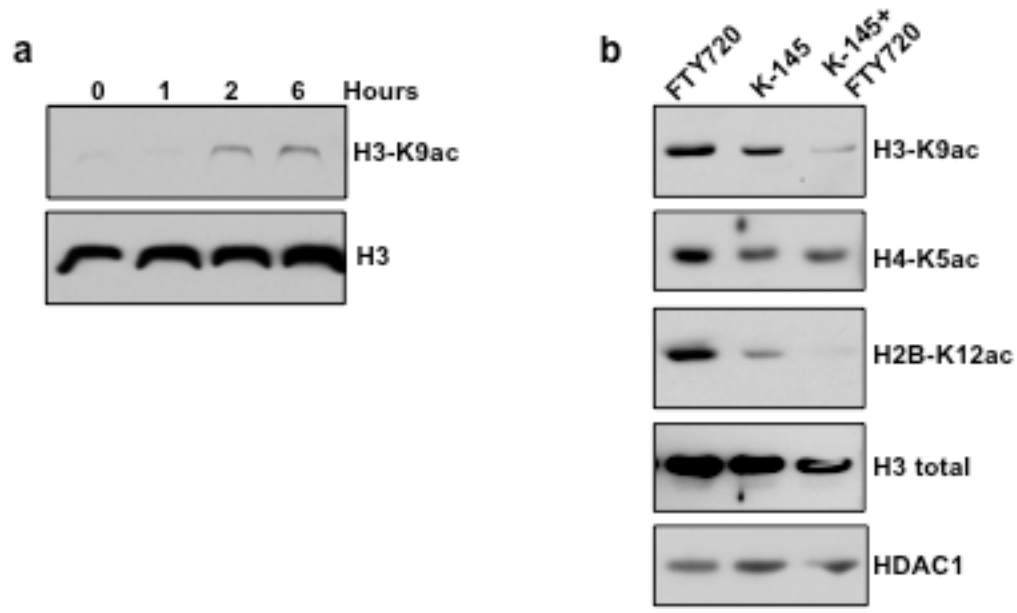
Supplementary Information accompanies this paper on the Oncogenesis website (http://www.nature.com/oncsis).

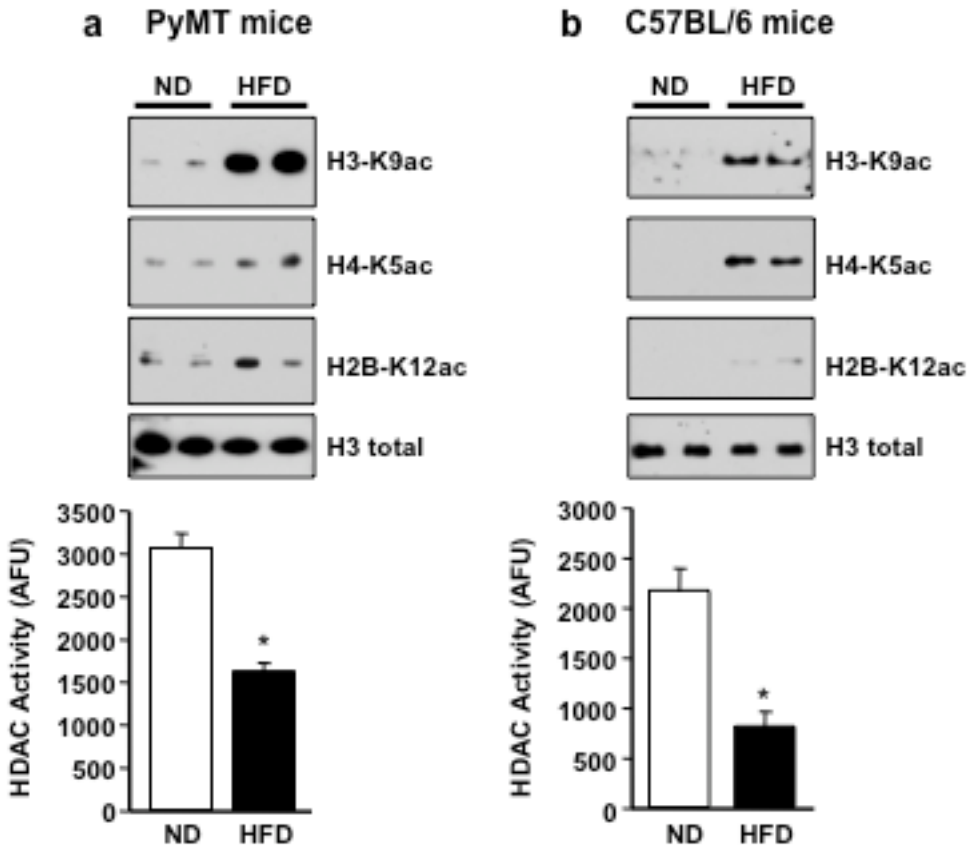
Supplementary Data



Supplementary Figure S1. FTY720-P inhibits activity of endogenous class I HDACs. Nuclear extracts of HeLa cells were immunoprecipitated with control IgG or the indicated HDAC-specific antibodies. Immunoprecipitates were washed, and HDAC activities were measured in the presence of vehicle, S1P (5 µM), FTY720-P (0.5 or 5 µM), FTY720 (5 µM), or SAHA (2 µM). Data are averages of triplicate determinations ± SD and expressed as arbitrary fluorescence units (AFU). *, $P < 0.01$, compared to Vehicle. Insets: Immunoprecipitates were analyzed by western blotting with HDAC-specific antibodies.



Supplementary Figure S2. FTY720 is phosphorylated by nuclear SphK2 in MDA-MB-231 and MCF7 human breast cancer cells. (a) MDA-MB-231 cells were treated with FTY720 (5 μM) for the indicated times. **(b)** MCF7 cells were pretreated with 1 μM K-145 for 30 min and then 1 μM FTY720 was added and the cells were incubated for an additional 4 h. Histone acetylations in nuclear extracts were detected by immunoblotting with the indicated antibodies.



Supplementary Figure S3. HFD consumption reduces HDAC activity and increases histone acetylation in tumor-free mouse mammary fat pads. Female PyMT transgenic mice (**a**) or naïve female C57BL/6 mice (**b**) were fed a normal diet (ND) or a HFD for 14 weeks and 15 months, respectively, and tumor-free mammary fat pads were removed. Nuclear extracts from mammary pads were analyzed by western blotting with the indicated antibodies. HDAC activity in nuclear extracts was determined and expressed as arbitrary fluorescence units. *, $P < 0.01$, compared to ND.

Sphingosine-1-Phosphate and Estrogen Signaling in Breast Cancer

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ABSTRACT

Breast cancer remains the most common malignant disease in women. The estrogen receptor-α (ERα) and its ligand 17β-estradiol (E_2) play important roles in breast cancer. E_2 elicits cellular effects by binding to ERα in the cytosol followed by receptor dimerization and translocation to the nucleus where it regulates gene expression by binding to ERE response elements. However, it has become apparent that E_2 also exerts rapid non-genomic effects through membrane-associated receptors. There is emerging evidence that this induces formation of the bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P). S1P in turn has been implicated in many processes important in breast cancer progression. One of the enzymes that produce S1P, sphingosine kinase 1 (SphK1), is upregulated in breast cancer and its expression has been correlated with poor prognosis. This review is focused on the role of the SphK/S1P axis in estrogen signaling and breast cancer progression and will discuss new therapeutic approaches targeting this axis for breast cancer treatment.

Keywords: sphingosine-1-phosphate, sphingosine kinase, estradiol, breast cancer, FTY720/Fingolimod.

Abbreviations: ERα, estrogen receptor α; EGFR, epidermal growth factor receptor; ERE, estrogen response element; ERK, extracellular signal regulated kinase; E_2, 17β-estradiol; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor 2; MAPK, mitogen activated protein kinase; PHB2, prohibitin 2; SphK, sphingosine kinase; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; TNBC, triple negative breast cancer; TRAF2, TNF receptor-associate factor 2.

INTRODUCTION

The estrogen receptor-α (ERα) and its ligand 17β-estradiol (E_2) play important roles in breast cancer. Most of the canonical genomic effects of binding of E_2 to ERα are mediated by nuclear transcriptional regulation. However, E_2 also exerts rapid non-genomic signaling through membrane-associated receptors many of them resulting from increased formation of the bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P). S1P and sphingosine kinases (SphKs) that produce it have been implicated in many processes important in breast cancer progression. In this review, we discuss the role of the SphK/S1P axis in estrogen signaling and breast cancer progression and also some new therapeutic approaches to potentially target this axis for breast cancer treatment.

Formation and metabolism of S1P

It has long been known that sphingolipid metabolism generates metabolites with important functions. The best characterized are ceramide, the backbone of all sphingolipids, its breakdown product sphingosine, and S1P. S1P metabolism has been discussed in many reviews (Hannun et al., 2008; Maceyka et al., 2014; Shamseddine et al., 2015) and is only briefly outlined here. Two sphingosine kinases, known as SphK1 and SphK2, catalyze the phosphorylation of sphingosine to S1P, which is irreversibly cleaved by S1P lyase to phosphoethanolamine and a fatty aldehyde or dephosphorylated back to sphingosine by several phosphatases which then can be reutilized for ceramide and sphingolipid formation. Tissue levels of S1P are thus determined by the balance between activity of SphKs and S1P lyase and phosphatases.

S1P signaling

S1P and its receptors

S1P has important roles in regulation of a wide variety of complex biological processes important for breast cancer progression (Carroll et al., 2015; Maceyka and Spiegel, 2014). Most of these actions are mediated by binding to a family of five specific cell surface receptors (S1PR1-5) (Maceyka and Spiegel, 2014). Numerous stimuli, including hormones such as estradiol (E_2), rapidly activate SphK1 and/or SphK2 to transiently increase intracellular S1P levels in specific pools. S1P produced mainly by activated SphK1 can then be secreted by Spns2, a member of the major facilitator superfamily of non-ATP-dependent transporters or by ABC transporters ABCA1, ABCC1, and ABCG2 (Nishi et al., 2014; Takabe et al., 2014). S1P in turn activates its receptors in an autocrine or paracrine manner known as 'inside-out' signaling of S1P (Hobson et al., 2001; Takabe et al., 2008). Physiological responses regulated by S1P depend on the spectrum of ubiquitously but differentially expressed S1PRs and the variety of G proteins they are coupled to. Thus, many signaling pathways downstream of S1PRs that have been linked to cancer progression have been shown to be activated depending on the cell type, including MAPKs, phospholipase C, adenylate cyclase, and Rac/PI3K/Akt, to name a few (Pyne et al., 2014; Takabe et al., 2008). Moreover, various types of cancer cells differentially express different sets of S1PRs, thus providing S1P with the ability to regulate numerous cellular processes important for breast cancer, including growth, survival, migration, invasion, inflammation, angiogenesis, and lymphangiogenesis (Nagahashi et al., 2014).

Intracellular actions of S1P

While it has long been suspected that S1P also has intracellular actions that are independent of S1PRs, only recently have several intracellular targets been identified that are likely to be important in the context of cancer. We found that S1P, but not dihydro-S1P, produced by SphK1 activated by TNF directly binds to and activates the E3 ubiquitin ligase activity of TNF receptor-associate factor 2 (TRAF2), an important component in NF-κB signaling (Alvarez et al., 2010). NF-κB regulates transcription of pro-survival or anti-apoptosis genes, thus identifying one of the mechanisms for the pro-survival actions of S1P in cancer progression. Interestingly, in contrast to SphK1, which is localized to the cytosol, SphK2 is mainly in the nucleus of most types of cells. We showed that nuclear S1P produced by ERK/MAPK-dependent activation of SphK2 is an endogenous inhibitor of histone deacetylases (HDACs) (Hait et al., 2009). Since SphK2 is present in repressor complexes together with HDACs in the nucleus of breast cancer cells (Hait et al., 2009), the S1P it produces inhibits HDAC activity resulting in enhanced transcription of specific target genes. This was the first indication that nuclear sphingolipid metabolism is involved in epigenetic regulation. Another link between nuclear S1P and gene expression was recently reported by Ogretmen and colleagues who discovered that S1P binds to hTERT and stabilizes telomerase at the nuclear periphery by allosterically mimicking hTERT phosphorylation. In murine xenografts, inhibitors of SphK2 decreased tumor growth and overexpression of wild-type hTERT in cancer cells, but not a hTERT mutant that was unable to bind S1P, restored tumor growth (Panneer Selvam et al., 2015). Their results suggest that S1P promotes telomerase stability and telomere maintenance important for cancer cell proliferation and tumor growth. In the mitochondria, SphK2 produces S1P that binds to prohibitin 2

(PHB2), a protein that regulates mitochondria assembly and function. Deleting SphK2 or PHB2 induced a mitochondrial respiration defect through cytochrome c oxidase (Strub et al., 2011) and may be important for the well-known Warburg metabolism of cancer cells. In this regard, it was suggested that SphK1, but not SphK2, functions to maintain the Warburg effect and cell survival (Watson et al., 2013).

Role of estrogen in breast cancer

Breast cancer is the most common cancer among women worldwide and occurs in about 1 in 8 women in the US (*http://www.cancer.org*, last accessed June 10, 2015). The estrogen receptor-α (ERα) plays an important role in breast cancer pathogenesis and progression (McDonnell et al., 2002). Patients with tumors that express ERα are termed "ERα-positive" and those lacking ERα are termed "ERα-negative". The majority of human breast cancers start out as estrogen-dependent because they are derived from cells that express ERα (Saha Roy et al., 2012). The steroid hormone, E_2, interacts directly with estrogen-specific cytoplasmic/nuclear receptors, ERα66 and ERα46 (Marino et al., 2006). ERα66 is the main ERα responsible for ERα-positive breast cancer responses to E_2. ERα46 is a 46 kDa splice variant that also functions in association with ERα66. The canonical pathway by which E_2 elicits cellular effects is initiated by binding to ERα in the cytosol followed by homo- or hetro-dimerization and translocation to the nucleus. In the nucleus, ERα dimers function as transcription factors by binding to specific response elements (ERE) on DNA, and either activating or repressing transcription (Mangelsdorf et al., 1995). These genomic responses are slow and take days to induce effects through translational events. E_2 also initiates rapid non-genomic responses, taking minutes to cause an effect through a membrane-associated

36 kDA splice variant (ERα36) of ERα66 (Marino et al., 2006), or through a G protein-coupled receptor (GPR30) (Filardo et al., 2007). Ample evidence has accumulated suggesting an important role for S1P in E_2-mediated signaling (Sukocheva et al., 2014).

Role of the SphK1/S1P axis in ER signaling

Our initial demonstration that overexpression of SphK1 in human breast cancer cells promoted tumorigenesis and neovascularization when implanted in nude mice (Nava et al., 2002) was followed by numerous reports confirming the importance of SphK1 and formation of S1P as anti-apoptotic and growth-promoting factors in breast cancer (Carroll et al., 2015; Newton et al., 2015; Pyne et al., 2014; Sukocheva and Wadham, 2014; Truman et al., 2014). Moreover, expression of SphK1 has been shown to correlate with poor prognosis in breast cancer patients (Pyne et al., 2014; Ruckhaberle et al., 2008).

E_2 activates SphK1 in breast cancer cells and increases formation and export of S1P (Sukocheva and Wadham, 2014; Takabe and Spiegel, 2014). Increased SphK1 activity correlates with enhanced cell growth, and also is required for E_2-dependent activation of MAPK and intracellular Ca^{2+} mobilization in ERα-positive MCF-7 breast cancer cells (Sukocheva et al., 2006).

Anti-estrogen therapy with tamoxifen or aromatase inhibitors is the treatment of choice for ERα-positive breast cancer. Unfortunately, loss of ERα expression leading to resistance to hormonal therapies is common and hormonal therapies are not effective in ERα-negative breast cancers. One of the mechanisms by which breast cancer cells become resistant is a switch to growth factor-dependent growth. Intriguingly, activation of ERα by E_2 activates EGFR by a "criss-cross" transactivation process important for

E_2-dependent growth that requires ERα, activation of SphK1, production and secretion of S1P that activates S1PR3, leading to enhanced processing of pro-EGFR and activation of EGFR (Sukocheva et al., 2006) (Figure 1). This also caused increased localization of EGFR in endosomes to delay its degradation and direct it for recycling for continuous proliferative signaling (Sukocheva et al., 2009). Interestingly, SphK1 mRNA is also increased after E_2 treatment, suggesting that SphK1 is transcriptionally regulated by ERα66. Transactivation to EGFR was also detected in T47D ERα-positive cells in response to E_2 treatment, but not in ERα-negative SK-BR-3; whereas, S1P was able to transactivate the EGFR in both ERα-positive and ERα-negative cells (Sukocheva et al., 2013). This suggests that both E_2 and S1P are critical components in the transactivation of EGFR in the transition from E_2-dependent growth to growth-factor-dependent growth. Moreover, SphK1 was required for EGF-induced breast cancer migration, proliferation, and cell survival and both ERα and GPR30 have been implicated in initiation of this signaling (Sukocheva and Wadham, 2014). However, only E_2 and not EGF stimulated export of S1P via ABCC1 and/or ABCG2 from breast cancer cells in an ERα-dependent manner (Takabe et al., 2010). Although Spns2, another bona fide S1P transporter, has been shown to export S1P from cells (Nishi et al., 2014; Takabe and Spiegel, 2014) and to be important in inflammatory and autoimmune diseases in mouse models (Donoviel et al., 2015), its involvement in breast cancer has not yet been investigated.

S1P in development of tamoxifen resistance

Tamoxifen is an anti-estrogen drug that binds to ERα, preventing estrogen binding, thereby causing cell growth arrest in breast cancer cells that are estrogen-dependent. Previous studies have shown that when patients with ERα-positive breast cancer are

treated with tamoxifen for 5 years, the rate of cancer recurrence is reduced by 39 percent and breast cancer mortality is decreased by about one-third throughout the first 15 years (Davies et al., 2011). Unfortunately, half of these patients will ultimately fail therapy due to acquired resistance. Moreover, breast cancer in patients whose tumors do not express ERα, progesterone receptor, and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2), termed triple-negative breast cancer (TNBC), is aggressive with high recurrence, metastatic, and mortality rates (Bayraktar et al., 2013). These patients do not respond to hormonal therapy due to *de novo* (intrinsic) resistance and have limited treatment options.

The SphK1/S1P/S1PRs axis has been implicated in the development of tamoxifen resistance or acquired (extrinsic) chemoresistance. SphK1 expression and activity were shown to be elevated in acquired-tamoxifen resistant ERα-positive MCF7 cells, and SphK1 inhibition or downregulation restored the anti-proliferative and pro-apoptotic effects of tamoxifen (Sukocheva et al., 2009; Watson et al., 2010). In a cohort of 304 ERα-positive breast cancer patients, SphK1 expression correlated with tamoxifen resistance (Pyne et al., 2012). Moreover, high SphK1 and ERK1/2 expression in tumors of ERα-positive breast cancer patients, high S1PR1, but not S1PR2, expression, and higher expression of S1PR1/3 and ERK1/2 were all found to be associated with shorter time to recurrence on tamoxifen (Watson et al., 2010). These correlations suggest that the SphK1/S1P/S1PR1/3 axis and ERK1/2 may cooperate to promote ERα-positive breast cancer progression and resistance to anti-estrogen therapies.

Inhibition of the ER/S1P axis

Inhibitors that effectively target the ERα/S1P axis could also potentially be useful as new therapies for breast cancer. Numerous studies have shown the inhibitors of SphK1 decrease cancer cell growth and survival and also sensitize them to chemotherapeutics. For example, the non-selective SphK1/2 inhibitor, SKI-II, has been shown to abrogate ERα signaling, likely acting both as a SphK inhibitor and in a similar manner as tamoxifen by directly binding to the ERα and blocking binding of E_2 (Antoon et al., 2011a). Moreover, SphK1 inhibition by siRNA knockdown or treatment with SKI-5C sensitizes TNBC cells to chemotherapeutic drugs (Datta et al., 2014). However, fewer studies have shown the utility of SphK1 inhibitors *in vivo*. We found that treatment mice bearing syngeneic breast tumors with the specific SphK1 inhibitor SK1-I not only suppressed S1P levels in the tumor and circulation, but importantly reduced tumor burden and metastases to lymph nodes and lungs (Nagahashi et al., 2012). Growth of MDA-MB-468 xenograft tumors in mice was significantly inhibited by the SphK1/2 inhibitor SKI-II and the tyrosine kinase inhibitor gefitinib when used in combination, but not as single agents (Martin et al., 2014). Although inhibition of SphK2 has also been reported to reduce tumorigenesis (Antoon et al., 2011b; Liu et al., 2013), further studies are needed to insure that these effects are solely dependent on inhibition of SphK2 activity.

We and others found that the multiple sclerosis pro-drug FTY720/fingolimod, a sphingosine analog that is phosphorylated mainly by SphK2 to a S1P mimetic *in vivo*, has pleiotropic anti-cancer actions in breast cancer cells and in animal models. First, FTY720 has anti-proliferative actions in many types of cancer cells without affecting normal cells (Romero Rosales et al., 2011). Moreover, FTY720 is a substrate and thus

a competitive inhibitor of SphK1 and SphK2, decreasing levels of pro-survival S1P and increasing levels of pro-apoptotic sphingosine (Pyne et al., 2014). Phosphorylated FTY720 (FTY720-P) is also a functional antagonist of and downregulates S1PR1, which interferes with activation of NF-κB and STAT3, and inhibits neovascularization in B cell-derived tumors (Deng et al., 2012; Lee et al., 2010; Liu et al., 2012) and colorectal cancer (Liang et al., 2013; Nagahashi et al., 2014). Furthermore, in breast cancer cells, FTY720-P produced in the nucleus by nuclear SphK2 is a potent inhibitor of class 1 HDACs that enhances histone acetylations and regulates expression of a restricted set of genes important for cancer progression, independently of its known effects on canonical signaling through S1PR1 (Hait et al., 2015). Importantly, in ERα-negative human and murine breast cancer cells and in ERα-negative syngeneic breast tumors, FTY720 activated re-expression of silenced ERα, which restored the ability of the anti-estrogen drug tamoxifen to block breast cancer proliferation and enhance apoptosis (Hait et al., 2015). Because a high fat diet and associated obesity are now endemic and associated with worse prognosis in breast cancer, we also investigated the effect of FTY720 administration on increased tumorigenesis in high fat diet fed mice. FTY720 significantly impaired development, progression and aggressiveness of spontaneous breast tumors in MMTV-PyMT transgenic mice and also reduced HDAC activity and restored expression of estrogen and progesterone receptors induced by the high fat diet (Hait et al., 2015). Taken together, these results provide further support the notion that FTY720 deserves consideration as a new therapeutic for treatment of both hormonal therapy-resistant breast cancer and triple-negative breast cancer.

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Competing interests statement

The authors declare no competing financial interests.

FIGURE LEGEND

Figure 1. Role of the SphK/S1P axis in signaling pathways initiated by E_2. Binding of E2 to cytosolic ERα induces its dimerization and translocation to the nucleus where it associates with estrogen response elements (ERE) to regulate gene expression. E_2 can also signal through cell surface E_2 receptors to initiate rapid non-genomic effects that include activation of SphK1 and production of S1P. After export of this S1P by transporters, it activates S1PRs (such as S1PR3) leading to downstream signaling that regulates many processes important for breast cancer progression including processing of pro-EGFR by metalloproteinases. EGF then stimulates EGFR-mediated signaling important for cell growth (Sukocheva et al., 2013). S1P produced in the nucleus by SphK2 is an endogenous inhibitor of HDACs (Hait et al., 2009).

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Genomic Pathway
Non-Genomic Pathway
EXTRACELLULAR
HDL
S1P
S1P
S1PR1-5
Metalloproteases
ERα
sphingosine
S1P
Transporter
pro-EGF
EGFR
ERα
ERα
S1P
G-proteins
Dimerization
SphK1
ERK1/2
Src
Akt
Proliferation
Angiogenesis
Chemoresistance
Migration
Survivability
CYTOPLASM
ERE
HDAC
S1P
NUCLEUS
Gene Expression
SphK2
Proliferation
Anti-Apoptosis

=17B-estradiol
= EGF
=stimulation/ activation
= inhibition